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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTI-BACTERIAL ACTIVITY. PART VI: THE BACTERICIDAL ACTION OF 8-HYDROXYQUINOLINE (OXINE).

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CERTAIN aspects of the antibacterial action of 8-hydroxyquinoline (oxine) have been described in Parts III and V of the present series (Albert, Rubbo, Goldacre and Balfour, 1947; Rubbo, Albert and Gibson, 1950). In Part III it was shown that the ability to form chelate complexes with divalent metals was the main factor determining activity. In Part V it was shown that the presence also of iron (or copper) in the medium was essential for this activity in the case of Gram-positive organisms. The hypothesis was put forward that oxine exerts its lethal effect by combining with these metallic ions in the medium, and that the complexes thus formed catalyze the oxidation of essential cell constituents. It was shown that cobalt can prevent injury to the cells, and it was suggested that this metallic ion may be an essential cell constituent whose function it is to protect, from oxidative destruction, a vitally important chemical group. The bacteriostatic action of oxine was also found to be antagonized by lowering the reduction potential of the medium or by adding intact red blood corpuscles.

All these effects were investigated by the usual *bacteriostatic* tests (turbidity after 48 hours' incubation at 37°). The present study deals with the *bactericidal* action towards *Staphylococcus aureus* of oxine in the presence of metallic ions. Thus it submits a representative portion of the territory previously explored to a closer and more quantitative scrutiny.

The following observations show that oxine is so rapidly bactericidal that it is unnecessary to postulate distinct bactericidal and bacteriostatic modes of action. Nevertheless only *dilute* solutions of oxine are rapidly bactericidal and, in the absence of traces of iron or copper, no antibacterial activity is exhibited at any concentration. A hypothesis has been devised to cover these facts, and also the reversal by cobalt of the toxic action of the metal-oxine complexes.

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EXPERIMENTAL.

The technique for demonstrating bactericidal action was modelled on that described by Miles and Misra (1938). This method, in the form used here, yields semi-quantitative results with great economy of media, and is especially suitable for performing many tests simultaneously under comparable conditions.

Solutions of reagents were prepared in glass-distilled water at ten times the final concentration required. Chemicals were of Analar quality. In critical experiments acid-washed Pyrex glassware was used to avoid contamination with extraneous metals. For the experiments where broth was the suspending fluid these precautions were unnecessary.

Inocula were prepared from 24-hour broth cultures appropriately diluted in broth or distilled water so that 0·15 ml. (5 drops) contained the organisms from 0·03 ml. of the original culture. When tests were made in metal-free solutions the cultures were centrifuged, twice washed in distilled water and re-suspended in distilled water.

Bactericidal activity was measured in the following manner. The test system was prepared by adding 1 ml. metallic salt solution and 1 ml. oxine solution to 8 ml. suspending fluid, and then inoculating with 5 drops of the diluted suspension by a standard dropping pipette. Sampling from the test system was also done using these standard pipettes. Before any sample was taken the organisms were evenly distributed by vigorous suction and expulsion with the sampling pipette. The sample, 0·06 ml. (2 drops), was then transferred to 5 ml. broth or water (depending on the nature of the suspending fluid), from which, after thorough mixing, two 0·03 ml. drops were placed on marked areas of a previously dried sheep blood-agar plate. The plates were then incubated for 48 hours at 37° and the degree of growth recorded as follows:

Confluent growth	++++
Discrete colonies, but uncountable	+++
50–150 colonies	++
10–50 colonies	+
1–10 colonies	±
No growth	—

RESULTS.

Initial experiments soon showed that oxine, dissolved in broth or peptone water, was markedly bactericidal in high dilution. For instance, an inoculum of approximately 5 million *Staph. aureus* was sterilized within one hour at room temperature by oxine at M/100,000, a concentration just sufficient to effect bacteriostasis. However, when these tests were repeated in media which had been substantially freed of the ions of heavy metals by previous treatment with oxine (see Rubbo *et al.*, 1950) no bactericidal activity was demonstrable. The result of a typical experiment in treated and untreated broth and in peptone water is recorded in Table I.

TABLE I.—*Bactericidal Action of Oxine in Various Media.*

Organism	*Staph. aureus.*
Oxine concentration	M/100,000.
Temperature	15°.
pH	7·0–7·2.

Suspending fluid.	Growth after exposure (hr.).				
	0.	1.	2.	3.	24.
Nutrient broth	+++	—	—	—	—
Peptone water 1 per cent	+++	—	—	—	—
Oxine-treated broth	+++	+++	+++	+++	+++

This observation added further support to our earlier conclusion that oxine is devoid of antibacterial action against Gram-positive bacteria unless traces of metallic ions are also present. As iron or copper ions were found to be most effective in this connection in the earlier work, they were tried in these bactericidal tests also. Tables II and III show the potentiating effect of ferrous, ferric and cupric ions on the bactericidal action of oxine in distilled water and in oxine-treated broth.

The high toxicity of cupric ions in distilled water makes it difficult to demonstrate their potentiating effect, but this can be readily shown in oxine-treated broth as indicated in Table III. Other metallic ions (calcium, magnesium, manganese, cadmium and cobalt) were tested, and found to be without marked

TABLE II.—*Bactericidal Action of Oxine in the Presence of Ferrous and Ferric Sulphates in Glass-distilled Water.*

Organism . . . . . *Staph. aureus.*
Temperature . . . . . 19°.
pH range . . . . . 6·0–6·8

Conc. of reagents added, 1/M.			Growth after exposure (hr.).			
Oxine.	$FeSO_4.7H_2O$.	$Fe_2(SO_4)_3.H_2O$.	0.	1.	3.	24.
nil	*nil*	*nil*	+++	+++	+++	+++
100,000	,,	,,	+++	+++	+++	+++
nil	100,000	,,	+++	+++	+++	+++
,,	*nil*	100,000	+++	+++	+++	+++
100,000	100,000	*nil*	+++	−	−	−
100,000	*nil*	100,000	+++	−	−	−

TABLE III.—*Bactericidal Action of Oxine in the Presence of Copper Sulphate in Glass-distilled Water and in Oxine-treated Broth.*

Organism . . . . . *Staph. aureus.*
Temperature . . . . . 15°.
pH range . . . . . 6·7–7·2.

Suspending fluid.	Conc. of reagents added.		Growth after exposure (hr.).			
	Oxine 1/M.	$CuSO_45H_2O$, 1/M.	0.	1.	3.	24.
Distilled water	50,000	*nil*	+++	+++	+++	+++
,, ,,	*nil*	100,000	+++	−	−	−
,, ,,	50,000	100,000	+++	−	−	−
Oxine-treated broth	50,000	*nil*	+++	+++	+++	+++
,, ,,	*nil*	100,000	+++	+++	+++	+++
,, ,,	50,000	100,000	+++	++	−	−

potentiating effect in distilled water. Zinc and nickel ions showed a slight potentiating activity but were not further investigated. Evidently the potentiation of oxine is not specific for a single metal.

It has been shown (Rubbo *et al.*, 1950) that cobaltous ions antagonize the growth-inhibitory effect of oxine in broth. From the results recorded here (Table IV) this antagonism can be attributed to the prevention by cobalt of the rapid bactericidal action of the iron-oxine and copper-oxine complexes. Table IV also shows that when cobalt antagonizes the action of oxine in the presence of copper, an excess of cobalt is required (Tube 6), whereas in the presence of iron one equivalent of cobalt is quite sufficient (Tube 3).

TABLE IV.—*Antagonism by Cobaltous Ions of the Bactericidal Action of Iron- and Copper-Oxine Complexes in Oxine-treated Broth.*

Organism	*Staph. aureus.*
Oxine concentration	M/25,000.
Temperature	20°.
pH	7·3.

Tube.	Conc. of metal salts added, 1/M.			Growth after exposure (hr.).			
	$FeSO_4.7H_2O$.	$CuSO_4.5H_2O$.	$CoSO_4.7H_2O$.	0.	2.	4.	24.
1	*nil*	*nil*	*nil*	+++	+++	+++	+++
2	50,000	,,	,,	+++	−	−	−
3	50,000	,,	50,000	+++	++	++	+++
4	*nil*	50,000	*nil*	+++	±	−	−
5	,,	50,000	50,000	+++	±	−	−
6	,,	50,000	10,000	+++	+++	+++	+++

Antagonism between cobalt and the iron-oxine complex can also be demonstrated under more clearly defined conditions in distilled water. It will be seen (Table V, Tubes 8 and 9) that at a low potentiating concentration of ferrous sulphate (Tube 8), protection can be obtained by the addition of excess cobalt sulphate (Tube 9). However, on increasing the iron concentration (Tubes 5 and 7) the same amount of cobalt fails to protect in spite of the fact that an excess of this metal is still present. This is not evident in the broth experiment where the cobalt ions are necessarily distributed between (*a*) the cells and oxine and (*b*) the amino-acids in the broth (for the affinities of amino-acids for the ions of heavy metals, see Albert, 1950, 1952).

TABLE V.—*Antagonism by Cobaltous Ions of the Bactericidal Action of the Iron-Oxine Complex in Glass-distilled Water.*

Organism	*Staph. aureus.*
Oxine concentration	M/100,000.
Temperature	20°.
pH range	6·8–6·9.

Tube.	Conc. of metal salts added, 1/M.		Growth after exposure (hr.).			
	$FeSO_4.7H_2O$.	$CoSO_4.7H_2O$.	0.	1.	2.	4.
1	*nil* (oxine control)	*nil*	+++	+++	+++	+++
2	100,000 (no oxine present: Fe^{++} control)	,,	+++	+++	+++	+++
3	*nil*	50,000 (Co^{++} control)	+++	++	++	+
4	100,000	*nil*	+++	−	−	−
5	100,000	50,000	+++	−	−	−
6	200,000	*nil*	+++	−	−	−
7	200,000	50,000	+++	+	−	−
8	400,000	*nil*	+++	−	−	−
9	400,000	50,000	+++	++	++	+

At this stage it was clear that the antibacterial action being studied did not involve a single substance, oxine, but resulted from the formation of various metal-oxine chelate complexes, the activity of which varied with the metal chelated.

Apart from the antagonism of this system by cobalt, a self-antagonism was discovered by studying the effect on bactericidal activity of increasing the concentration of oxine. The results showed a strikingly paradoxical effect; increasing

the concentration of oxine progressively decreased the rate of kill. This unexpectedly inverted type of action is shown in Table VI. It will be seen that the efficiency and speed of the bactericidal action is greatest at the lowest inhibitory concentration (M/102,400), and least at the highest concentration (M/800). It is evident, however, that there is a slow bactericidal action proceeding at these higher concentrations, which are, of course, bacteriostatic.

It was soon found that this inverted bactericidal effect depends upon the presence of an *excess of oxine* over the potentiating metal available in the medium. As shown in Table VII, the addition of 4 equivalents of ferrous sulphate to each oxine concentration restores rapid and complete bactericidal activity. It is

TABLE VI.—*Effect of Increasing Concentration on the Bactericidal Action of Oxine in Broth.*

Organism . . . . *Staph. aureus.*
Temperature . . . . 20°.
pH range . . . . 7·0–7·3.

Concentration of oxine, 1/M.	Growth after exposure (hr.). 0.	1.	3.	24.	Growth on incubation, 24 hr.; bacteriostatic test.
800	+++	+++	+++	+	*nil*
1,600	+++	+++	+++	+	,,
3,200	+++	+++	+	+	,,
6,400	+++	+++	+	−	,,
12,800	+++	+	+	−	,,
25,600	+++	+	±	−	,,
51,200	+++	+	−	−	,,
102,400	+++	−	−	−	,,
204,800	+++	+++	+++	+++	Heavy

therefore obvious that the concentration of oxine must be carefully adjusted in relation to the metallic ions available in order to secure maximal antibacterial activity.

TABLE VII.—*The Effect of Added Ferrous Sulphate on the Inverted Bactericidal Action of High Concentrations of Oxine in Broth.*

Organism . . . . *Staph. aureus.*
Temperature . . . . 22°.
pH . . . . . 7·3.

Tube.	Conc. of reagents added, 1/M. Oxine.	$FeSO_4.7H_2O$.	Growth after exposure (hr.). 0.	1.	3.	24.
1	*nil*	400	+++	+++	+++	++++
2	800	*nil*	+++	+++	+++	+
3	800	400	+++	−	−	−
4	1,600	*nil*	+++	+++	++	±
5	1,600	800	+++	−	−	−
6	3,200	*nil*	+++	+++	++	±
7	3,200	1,600	+++	−	−	−
8	12,800	*nil*	+++	+	±	−
9	12,800	6,400	+++	−	−	−

Conversely, it has been found that an *excess of metal* also will reduce the bactericidal rate in broth, but shows no effect on the bacteriostatic end-point as was the case with excess oxine. The results in Table VIII show an additive degree of protection occurring with increasing iron concentration when the

oxine level remains fixed. This effect can be readily neutralized by increasing the oxine concentration to equivalence with that of the ferrous salt.

TABLE VIII.—*The Effect of Added Ferrous Sulphate on the Bactericidal Action of a Low Concentration of Oxine in Broth.*

Organism	*Staph. aureus.*
Oxine concentration	M/100,000.
Temperature	17°.
pH range	6·8–7·2.

Conc. of $FeSO_4.7H_2O$ added, 1/M.	Growth after exposure (hr.). 0.	1.	3.	24.
nil	+++	−	−	−
50,000 (4 equivalents)	+++	±	±	±
5,000 (40 „)	+++	++	+	+
2,000 (100 „)	+++	+++	+++	+++

Further experiments of a type similar to those described in Table VIII were made with other metallic ions in order to discover whether this antagonism was specific for ferrous ion. The results are listed in Table IX, in which it will be seen that a *large excess* (200 equivalents) of ferric, zinc, nickel, cobalt or cadmium ions in the medium effectively antagonizes the bactericidal action in broth. However, cobalt is the only metal (in this series) of which *one equivalent* can antagonize the action of oxine (cf. Table IV). In contrast to this high efficiency of cobalt, those metallic ions which form only weakly-bound complexes with oxine (namely calcium, magnesium and manganese) are without effect.

TABLE IX.—*Antagonism of the Bactericidal Action of Oxine by Addition of an Excess of Various Metallic Salts.*

Medium	Broth.
Organism	*Staph. aureus.*
Oxine concentration	M/100,000.
Temperature	18°.
pH range	6·6–7·2.

Conc. of added metal salt* (200 equivalents) M/1000.	Growth after exposure (hr.). 0.	2.	4.	24.
None added	+++	−	−	−
Calcium chloride	+++	−	−	−
Magnesium sulphate	+++	−	−	−
Manganous sulphate	+++	−	−	−
Cadmium sulphate	+++	+++	++	+
Ferrous sulphate	+++	±	±	±
Cobaltous sulphate	+++	+++	+++	++
Zinc sulphate	+++	+++	+++	+++
Nickel sulphate	+++	+++	+++	+++
Ferric sulphate	+++	+++	+++	++++

* These salts are listed in order of their increasing affinity for oxine.

DISCUSSION.

Our present and previous findings make it clear that the mode of action of oxine depends on its chelating powers. It is equally clear that this action is not a simple one. The facts are as follows : Oxine, in high dilution, is rapidly bactericidal to Gram-positive organisms (as exemplified by *Staph. aureus*), and its

growth-inhibitory activity is evidently a manifestation of this bactericidal action. However, as this action is entirely dependent upon the presence of one or more of three metallic ions (cupric, ferrous and ferric), it can be concluded that these metal-oxine complexes are the true toxic agents (Tables I, II and III). Anderson and Swaby (1951) have recently shown that the fungistatic action of oxine also depends on the presence of ferrous or cupric ions.

It has also been shown that the relative concentrations of oxine and metallic ions cannot be varied beyond a fairly restricted limit without resulting in a depression of bactericidal action, the degree of which depends on the disproportion thus created. For example, a M/100,000 solution of oxine in broth kills in approximately one hour, but a 125-fold stronger solution (M/800) kills so slowly that viable organisms can still be demonstrated after 24 hours (Table VI). This is surely one of the most paradoxical effects yet demonstrated in the whole literature of disinfection. That this effect is caused by chemical imbalance is shown by the fact that the addition of iron salts to the medium (which already contains enough iron for the M/100,000 solution of oxine to function) permits the M/800 solution to become rapidly bactericidal (cf. Tables I and VII). The effects of imbalance have also been demonstrated by the addition of an excess of ferrous sulphate (M/5000 or more) to broth. In this case the rapid bactericidal action of M/100,000 oxine is so retarded that viable organisms can be demonstrated after 24 hours' exposure (Table VIII). Thus we see that an excess of either of the reactants greatly decreases the rate of bactericidal activity without eliminating it. These facts were not discoverable by a study of bacteriostatic action, because the technique used could only measure an all-or-nothing effect, not a graded response.

Any hypothesis to cover these observed phenomena must also take account of two other observations: (*a*) Low concentrations of cobaltous ions antagonize the lethal action of iron-oxine and copper-oxine in broth and in distilled water (Tables IV and V); (*b*) other metallic ions can bring about this result, but only in considerably higher concentrations (Table IX). The hypothesis which we offer is based on the law of mass action and the known chemistry of oxine.

Chemical aspects.

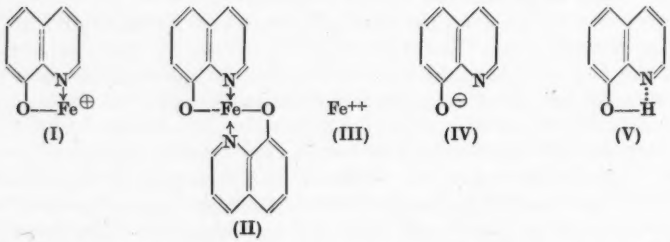
So that this hypothesis may be more clearly understood, some quantitative aspects of the chelating properties of oxine will first be reviewed. Divalent metallic ions form two kinds of complex with oxine, a 1:1 complex (*e.g.*, I) in which one metallic cation is bound (by chelation) to only one oxine anion, and a 1:2 complex (*e.g.*, II) in which the metallic ion is saturated by combination with two oxine anions. Complexes of type (II) are freely in equilibrium with those of type (I), and both are in equilibrium with free metallic ions (III) and free oxine anions (IV). The proportions of (I), (II), (III) and (IV) which will be present under various conditions is determinable from the stability constants for these complexes, as given by the following equations and recorded in Table X.

$$\log K' = \log [I] - \log [Fe^{++}] - \log [\text{oxine anions}] \quad . \quad . \quad \text{(i)}$$
$$\log K'' = \log [II] - \log [I] - \log [\text{oxine anions}] \quad . \quad . \quad \text{(ii)}$$

The values in Table X were obtained by potentiometric titration (Albert, 1953).

TABLE X.—*Comparative Avidity of Oxine for the Ions of Metals.*

Cation.	Log K′.	Log K″.	Logarithm of overall stability constant (K′K″).
Ca^{++}	3·5	—	—
Mg^{++}	4·5	—	—
Mn^{++}	6·8	5·8	12·6
Cd^{++}	7·2	6·2	13·4
Fe^{++}	8·0	7·0	15·0
Co^{++}	9·1	8·1	17·2
Zn^{++}	(approximately same as Co^{++})		
Ni^{++}	9·9	8·8	18·7
Cu^{++}	12·2	11·2	23·4
Fe^{+++}	12·3	11·3	(K′K″K‴) 33·9



From equations (i) and (ii) it is evident that the law of mass action operates in such a way that an excess of metallic ions will favour (I) at the expense of (II) and (IV), whereas an excess of oxine (V) will favour (IV), and hence (II), at the expense of (I) and (III). (For a fuller discussion of the mathematics of metal-complex equilibria, see Albert, 1950, 1952.)

Complexes of the types (I) and (II) differ greatly in their physico-chemical properties: type (I) is positively charged, freely soluble in water and almost insoluble in lipoids and lipoid-solvents, whereas type (II) is electro-chemically neutral, freely soluble in lipoids and lipoid-solvents but almost insoluble in water. At pH 7 oxine is in the non-ionized state to the extent of 99 per cent and is soluble in both lipoids and water.

Trivalent metallic ions (*e.g.*, Fe^{+++}) form three types of complex, a 1:1 complex which carries two positive charges, a 1:2 complex which carries a single positive charge (both of these complexes are freely soluble in water, but not in lipoids), and a 1:3 complex (Berg, 1929) which is soluble in lipoids but not in water.

That oxine is not toxic in the cell, where it can be assumed to penetrate, must be ascribed to the presence therein of substances having greater affinity for the metabolically important metals (cf. the very high affinity of cysteine for cobalt (Albert, 1952)).

The hypothesis will now be developed that the iron-oxine complex (I) exercises an antibacterial action by combining with metal-binding groups which form part of an essential bacterial enzyme or metabolic system.

Hypothesis of mode of action.

From these facts we pass to the biological data, which show that the conditions under which oxine is most actively bactericidal are those in which a gross

excess of neither oxine nor of potentiating metal is present (Tables VI and VIII). (At the same time it must be noted that although an excess of either reagent greatly reduces antibacterial activity a trace of activity does persist, and it can be readily shown that growth is somewhat inhibited.) Such findings can best be explained by assuming that the type (I) complex is toxic whereas the type (II) is not.

The validity of such a view can be tested on chemical and bacteriological grounds. Chemically, the 1:2 iron-oxine complex (type II) is saturated in respect of its chelating properties, and therefore cannot combine with further molecules such as biologically important metal-binding substances. On the other hand, the 1:1 complex (type I) is unsaturated, and hence is capable of further combination with metal-binding constituents of the cell. The poor bactericidal activity shown in high concentrations of oxine (Table VI) is attributable to the excess of oxine over available metal because these proportions favour the formation of a relatively large proportion of type (II) complex (law of mass action). It is thus that the apparently paradoxical effect arises where an increase in the concentration of oxine leads to a decrease in antibacterial action. However, the addition of iron to such a system restores the rapid bactericidal activity by shifting the chemical equilibrium towards formation of 1:1 complexes (Table VII). These results clearly indicate that the 1:1 iron-oxine complex is the actual cytotoxic agent.

The antibacterial action of this complex can be antagonized by the addition of 1–2 equivalents of cobalt (in relation to the oxine concentration ; Table IV), or by adding a large excess (100 to 200 equivalents) of such metallic ions as ferrous, nickel, zinc or cadmium (Table IX). The specific antagonism by Co^{++} in trace amounts has previously been noted (Rubbo *et al.*, 1950).

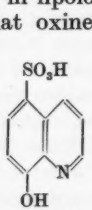
The antagonism by trace-amounts of cobalt might suggest that the type (I) complex is acting as an antimetabolite competing with a normal metabolite, *viz.*, cobalt ion. Although the two ions (iron-oxine and Co^{++}) are dissimilar in shape, mass and charge, nevertheless metallic ions inside the cell are bound by amino-acids, purines, etc. : cobalt bound as a 1:1 complex in this way would be not unlike the iron-oxine complex. On the other hand, it might be suggested that the type (I) complex is competing with a complex cobalt metabolite. That this metabolite is not vitamin B_{12} was proved experimentally by adding 0·01–20μg./ml. of vitamin B_{12} to broth containing M/50,000 and M/100,000 oxine : no prevention of antibacterial action was noted. An earlier observation (Rubbo *et al.*, 1950) that oxine action can be antagonized by lowering the oxidation-reduction potential leads us to suggest that added cobalt may be able to stabilize an — SH enzyme system which is otherwise oxidatively destroyed by the type (I) iron- or copper-oxine complexes. This suggested mechanism has a simple precedent in the work of Baur and Preis (1936), who showed, *in vitro*, that the oxidative destruction of cysteine (when catalysed by Cu^{++}) is inhibited if 20 times as much Co^{++} as Cu^{++} is present (there is no Co^{++} : Fe^{++} antagonism in this system). It is possible that the final toxic agent inside the cell is not the type (1) complex, but actually inorganic iron or copper liberated from the complex by metabolic oxidation. However, there is one piece of evidence that iron-oxine can be an oxidation catalyst in its own right ; a mixture of Fe^{++} and oxine (but not Fe^{++} alone) has been found to oxidize the — SH groups in nucleoproteins from rat liver and from fish eggs (Bernheim and Bernheim, 1939).

Streptomycin is another antibacterial whose action is inhibited when the oxidation-reduction potential is lowered (Bondi, Dietz and Spaulding, 1946). However, the modes of action of streptomycin and oxine have, so far as is known, nothing in common.

In regard to the non-specific antagonism by other divalent metals, that caused by Fe^{++} (Table VIII) is of greatest interest because this metal is required to potentiate the antibacterial action. It seems likely that excess Fe^{++} slows the bactericidal rate by repressing the formation of the 1:2 iron-oxine complex (law of mass action). The significance of this repression becomes clear if it is assumed that the site of action of the type (I) complex is intracellular, and that it is transported across the cell membrane principally as the type (II) complex. This suggestion requires some elaboration.

As indicated earlier, the essential differences between type (I) and type (II) complexes are that the former is positively charged (and non-liposoluble), whereas the latter, like oxine itself, is neutral (and liposoluble). It is reasonable to assume from the work of Davson and Danielli (1943) that the penetration of type (I) complexes through the cytoplasmic membranes of bacteria (which according to Knaysi, 1951, are lipoprotein complexes) would be very considerably slower than that of the type (II). This difference in rate of penetration would be detected by measuring the rate of kill rather than inhibition of growth. When oxine is in large excess over the potentiating metal (Table VI), both the type (II) complex and oxine would predominate and would be expected to diffuse rapidly across the cell membrane. However, the excess of oxine within the cell would prevent the type (II) complex from breaking down into the toxic type (I) by the mass action effect. When the ratio of oxine and Fe^{++} is more nearly balanced, the type (II) complex, unaccompanied by an excess of oxine, would diffuse into the cell, and there, according to the law of mass action, some type (I) complex must be formed from it. Thus the type (II) complex could act as a vector (or carrier) for the type (I) complex. When Fe^{++} is in excess, the slow diffusing type (I) complex predominates, resulting in a far slower bactericidal action.

The assumption that the action of oxine takes place within the cell (see p. 126), could be tested by using a derivative of oxine which forms non-liposoluble type (II) complexes. Oxine-sulphonic acid (VI) is such a substance. Its avidities for the various metallic ions are identical with those of oxine (Albert, 1953), but these complexes do not dissolve in lipoids and lipoid-solvents. It is, therefore, of special interest to note that oxine-sulphonic acid lacks antibacterial properties (Albert *et al.*, 1947).



(VI)

A different reason can be advanced for the antagonistic action of heavy metal ions other than iron, notably Ni^{++} and Zn^{++}, when they are added in great excess (200 equivalents) to the medium (Table IX). These metallic ions do not potentiate the action of oxine (Rubbo *et al.*, 1950), hence their complexes with oxine are relatively non-toxic. From our knowledge of the comparative avidity

of metallic ions for oxine (Table X) it is clear that these metals can successfully compete with Fe^{++} (and, if in sufficient excess, with Cu^{++} ions) for oxine, and by combining with it, prevent the formation of the toxic iron-oxine complex. This would be expected to prevent the action of oxine, as shown in Table IX. It should be noted that equal quantities of the various metallic ions antagonize oxine roughly in proportion to their affinity for it, which is (in increasing order) Ca^{++}, Mg^{++}, Mn^{++}, Cd^{++}, Fe^{++}, Co^{++} (= Zn^{++}), Ni^{++}, Cu^{++}, Fe^{+++}.

From knowledge of the comparative avidity of metallic ions for oxine (Table X), it is evident that (in circumstances such as those of Table IX when 1:1 complexes predominate), it would take only a 6-fold excess of Cd^{++} to begin to compete effectively with Fe^{++}, but a 100,000-fold excess to compete with Fe^{+++} or Cu^{++}. Similarly, Ni^{++} could begin to compete effectively with an 80-fold excess of Fe^{++}, but a 250-fold excess of Ni^{++} would be required to have the same effect on Fe^{+++} or Cu^{++}. Such calculations can be only approximate because they do not take account of competition from other chelating substances in broth and cells. Nevertheless it would be a fair assumption from the results in Table IX that much of the "potentiating metal" in the medium is Fe^{++}, and not Fe^{+++} or Cu^{++}, because 200 equivalents of cadmium are almost able to overcome the potentiating effect.

Such arguments cannot be used to explain antagonism by Co^{++}, although it has an 11-fold higher affinity for oxine than Fe^{++} (Table X). We have already shown (Rubbo *et al.*, 1950) that Ni^{++}, which has a greater affinity for oxine than Co^{++}, cannot antagonize the action of oxine, when 5 equivalents (or less) are added to the medium, but Co^{++} readily does so. Moreover, it is evident from Table X that 5 equivalents of Co^{++} could not displace more than 1/1000th part of the Cu^{++} in copper-oxine, but this amount can promote growth in the presence of the copper-oxine complex (Table IV). We therefore conclude that Co^{++} antagonism is qualitatively different from that shown by other metals.

This discussion would not be complete without a brief reference to the work of Feeney (1951) on the antibacterial action of conalbumin. It is claimed that this protein, which firmly binds Fe^{++}, exerts its antibacterial action by removing Fe^{++} from the cells (into which it cannot diffuse), thus causing an imbalance in the Fe^{++} : Co^{++} ratio which proves fatal through cobalt-poisoning. Seeking a parallel between the action of this protein and that of oxine, Feeney goes on to suggest that oxine also acts outside the cell, first removing all Co^{++} from the cell and then (when used in conjunction with Fe^{++}) causing an imbalance in the Fe^{++} : Co^{++} ratio which proves fatal through iron-poisoning. That this attempted explanation is incorrect is shown (*i*) by the ever-increasing evidence that the site of action of oxine is inside the cell; (*ii*) because media which have been rendered poor in metallic ions by extraction with oxine are not toxic to bacteria, nor do they become so when Co^{++} or Fe^{++} are added together or separately, nor when oxine is added, but the addition of both Fe^{++} and oxine is rapidly fatal to the bacteria (Tables III and IV). Thus a ferrous-oxine complex is the true toxic agent.

SUMMARY.

Investigation of the action of oxine (8-hydroxyquinoline) on *Staph. aureus* showed that oxine is bactericidal at the dilutions found effective in bacteriostatic tests.

Whereas oxine is rapidly bactericidal at a dilution of M/100,000, stronger solutions (*e.g.*, M/800) have little action (Table VI).

When tested in distilled water (or in broth where the concentration of heavy metals had been lowered below a critical value), oxine had no bactericidal action at any dilution (Tables I and II). In such media the bactericidal activity was restored by the addition of traces of iron or copper salts (Tables II and III).

Bactericidal action depends upon a balanced ratio between oxine and iron in the medium. Even M/800 solutions of oxine become rapidly bactericidal when the iron content is raised (Table VII). On the other hand, M/100,000 solutions cease to be bactericidal when too much iron is present (Table VIII).

Cobalt is unique in that as little as one equivalent can antagonize the antibacterial action of these oxine-metal combinations (Table IV), whereas great excesses of other metals are required (Table IX).

The following hypothesis is suggested to cover these facts :

(*a*) Of the two species of ferrous-oxine complexes, the 1:1 complex (which is formed when iron is in excess and which has an affinity for metal-binding groups) is antibacterial. The 1:2 complex (which is the main product when oxine is in excess and, being saturated, cannot act chemically like the 1:1 complex) is devoid of antibacterial activity, like oxine itself.

(*b*) The site of action of the 1:1 complex appears to be intracellular. Although slow to penetrate, the 1:1 complex can be formed within the cell from the rapidly-penetrating 1:2 complex provided that a favourable ratio of oxine to iron is maintained.

The rôle played by cobalt in antagonizing the action of the oxine-metal complexes is discussed in relation to a simple model experiment carried out *in vitro*.

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OBSERVATIONS ON HAEMAGGLUTININ "LINKAGE" IN RELATION TO ISO-AGGLUTININS AND AUTO-AGGLUTININS.

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LINKAGE of anti-A and anti-B agglutinins in O sera was observed by Landsteiner and Witt (1926). When either anti-A or anti-B is absorbed with the homologous agglutinogen a fall in titre of the heterologous agglutinin sometimes results. That this is not due to non-specific absorption is shown by the fact that heterologous absorption does not occur with A or B sera.

Harley (1936) described a serum in which the linkage occurred in one direction only. The B——anti-B absorption was strictly specific but the A——anti-A absorption showed a heterologous effect.

Dodd (1952) confirmed the existence of anti-A——anti-B linkage in certain O sera and showed that antibody solutions prepared by warming A or B cells after they had absorbed antibody from these sera could agglutinate both A and B cells. She concluded that such sera must contain antibody molecules with both anti-A and anti-B-combining sites or receptors.

Bird (1951*a*) described a serum (Group AB) which contained powerful auto-agglutinins. These were of interest, because if completely absorbed with the patient's own cells their action against other cells disappeared, but if absorbed with O cells action against A, B, and the patient's cells persisted. If the serum was further absorbed with A cells action against B and the patient's cells persisted, and if then absorbed with B cells the serum acted weakly against the patient's cells. He considered this to indicate that auto-agglutinins might be a combination of multiple specific agglutinins which could be selectively absorbed. Some sera containing auto-agglutinins active only in the cold behaved in the same way.

This phenomenon is not reproducible in many instances. A serum (Group A) containing powerful auto-agglutinins was recently examined in the same manner as the one described above (Bird, unpublished observations). After complete absorption with O cells, action against A cells and the patient's own cells was considerably reduced. With most other sera containing auto-agglutinins active only in the cold, absorption with O cells results in removal of all agglutinins. In fact, the concept of non-specificity of auto-agglutinins is based on such results. These differences in the behaviour of auto-agglutinins in various sera may be due to the occurrence of a greater or lesser degree of linkage. The present investigation attempts to confirm previous observations on iso-agglutinin linkage and to determine if auto-agglutinins consist of similarly linked antibodies.

EXPERIMENTAL.

In the present study, O sera obtained from random Indian blood donors were first examined. The object of this preliminary study of iso-agglutinins was to observe whether the occurrence of anti-A and anti-B linkage conformed to any set pattern. The donors had had no previous

transfusions or injections of blood, and gave no history of serious illness in the past. The anti-A and anti-B agglutinins were titrated by the two-fold dilution method. Two per cent suspensions of well-washed fresh A and B cells obtained from the same persons were used throughout. Each serum was divided into two portions. From one portion the anti-A agglutinin was completely neutralised by the addition of A-specific substance. Then the anti-B agglutinin was titrated. From the other portion the anti-B agglutinin was completely inactivated by B-specific substance and then the titre of the anti-A agglutinin was determined. The group specific substance used in each case was dried ovarian cyst fluid. A minute amount was sufficient to neutralise the corresponding antibody ; hence no significant dilution of the serum resulted. The results are shown in Table I. Further samples of those sera in which linkage appeared to be present were then absorbed with A or B cells, depending on the " direction " of linkage observed in the experiments with A and B substance. The cells used for absorption were then thoroughly washed at about 4°, and the absorbed antibody eluted with saline in a bath at 54° using the technique of Schiff and Boyd (1942). The results are shown in Table II. As a control, similar absorptions were carried out in each series with a mixture of A and B sera.

Sera containing auto-agglutinins (" non-specific " cold agglutinins) were titrated against various cells at 4°, at laboratory temperature, and at 37°. Some of those which acted specifically at higher temperatures were selected for further examination. These sera were absorbed with appropriate O cells at 4° and their action against various cells was determined. The absorbed antibody was eluted from the O cells with warm saline as before. The object of examining auto-agglutinins in this manner was to determine if the variable results referred to in the introduction could be due to linkage. The results obtained with the various selected sera are shown in Table III. In addition, as a control, iso-agglutinins in various sera containing cold auto-agglutinins were titrated before and after absorption with O cells in the cold. The results are shown in Table IV.

DISCUSSION.

Anti-A——anti-B and anti-B——anti-A linkage is present in samples 4, 6, 15, 18 and 22 (Table I).

Linkage in one direction only is present in samples 5, 11, 14, 16, 19, 20, 23, 24, 27, 29, 30, 31 and 32 (anti-A——anti-B), and in samples 1, 7 and 26 (anti-B——anti-A). Elution confirmed the occurrence of linkage in all these samples except 19 and 29.

Samples, 2, 3, 8, 9, 10, 12, 13, 17, 21, 25 and 28 showed no linkage in either direction. In the above assessment a 1-tube fall in titre has been ignored. Sample 3 showed a prozone phenomenon, there being no agglutination up to 1 in 4 in the case of anti-A and up to 1 in 2 in the case of anti-B. After the absorptions there was no fall in titre, but the prozone disappeared. This is shown more clearly in Table V.

The disappearance of the prozone from sample 3 is worthy of consideration. The prozone in this serum is evidently not due to a gross excess of antibody because the titres are within normal limits, and the antigen strengths are constant for the whole series. It may be that the phenomenon resulted from the presence of incomplete antibody, but this is unlikely, since none of the donors concerned had had a previous transfusion or injection of blood, or injection of horse serum, and the titres of sample 3 against cells suspended in albumin were no higher than with those suspended in saline.

It is apparent from Tables I and II that the occurrence, degree, or " direction " of iso-agglutinin linkage are variable and do not seem to obey any set laws. The circumstances under which dual-receptor iso-antibodies are formed in some sera and not in others await elucidation, and there is as yet no explanation for linkage in one direction only.

TABLE I.—*Anti-A and Anti-B Titres Before and After Neutralisation with A and B Substance.*

Sample number.	Titre before neutralisation.		Titre after neutralisation. of anti-A.		Titre after neutralisation. of anti-B.	
	Anti-A.	Anti-B.	Anti-A.	Anti-B.	Anti-A.	Anti-B.
1	256	128	0	64	64	0
2	256	32	0	16	128	0
3	64	64	0	64	64	0
4	256	128	0	4	32	0
5	16	16	0	2	8	0
6	512	512	0	128	32	0
7	256	256	0	256	32	0
8	256	256	0	256	128	0
9	128	32	0	16	64	0
10	8	4	0	2	8	0
11	128	64	0	8	64	0
12	256	32	0	16	256	0
13	128	32	0	32	64	0
14	64	16	0	4	64	0
15	64	64	0	16	16	0
16	128	64	0	16	128	0
17	64	64	0	64	64	0
18	2048	16	0	8	512	0
19	64	64	0	16	64	0
20	128	32	0	4	128	0
21	128	16	0	8	128	0
22	512	512	0	8	32	0
23	128	32	0	8	64	0
24	64	64	0	16	64	0
25	64	16	0	8	64	0
26	8192	4096	0	2048	1024	0
27	256	256	0	64	256	0
28	128	128	0	128	64	0
29	32	16	0	4	32	0
30	256	64	0	16	256	0
31	32	32	0	4	32	0
32	128	128	0	32	128	0
Control (*A Serum* + *B serum*):						
X	64	64	0	64	64	0
Y	128	64	0	64	128	0
Z	64	64	0	64	64	0

Sera 1 and 5 of Table III appear to be examples of auto-agglutinin linkage. At room temperature only A_1 cells were agglutinated by these sera. This would ordinarily be taken to indicate that the sera contained both non-specific as well

TABLE II.—*Anti-A and Anti-B Titres Before and After Absorption with A or B Cells and Activity of Eluted Antibody.*

Sample number.	Cells used for absorption.	Titre before absorption.		Titre after absorption.		Activity of agglutinin solution.	
		Anti-A.	Anti-B.	Anti-A.	Anti-B.	Anti-A.	Anti-B.
4	A	256	128	0	2	+	+
6	B	512	512	16	0	+	+
7	B	256	256	16	0	+	+
14	A	64	16	0	4	+	W
15	A	64	64	0	16	+	+
16	A	128	64	0	16	+	+
18	B	2048	16	256	0	W	+
19	A	64	64	0	8	+	—
20	A	128	32	0	4	+	W
22	A	512	512	0	8	+	W
24	A	64	64	0	8	+	W
27	A	256	256	0	64	+	W
29	A	32	16	0	4	+	—
30	A	256	64	0	8	+	W
31	A	32	32	0	4	+	W
32	A	128	128	0	32	+	W
Control (*A serum* + *B serum*):							
X	A	64	64	0	64	+	—
Y	A	128	64	0	64	+	—
Z	A	64	64	0	64	+	—

Samples 1, 5, 11, 23 and 26 were not examined.
+ = distinct agglutination.
W = weak ,,

as specific (anti-A_1) cold agglutinins. In the light of the concept of multiple specificity of auto-agglutinins (Bird, 1951*a*), this could also indicate that the anti-A_1 auto-agglutinin component was more powerful than the other components. When these sera were absorbed with O cells in the cold, anti-A_1 activity persisted to a lesser extent than before absorption. The anti-A_1 antibodies were now weakly active at room temperature, whereas unabsorbed control sera still strongly agglutinated other samples of the same A_1 cells. The anti-A_1 titre in the cold was considerably reduced. This could be a linkage effect, involving both the anti-A_1 agglutinins active at room temperature and those active only in the cold. In no case was there such a fall in titre of iso-agglutinins (Table IV). Elution revealed that all components had been absorbed together. With the other sera containing auto-agglutinins elution experiments were inconclusive.

The alternative explanation for these results is that a single agglutinin is involved. If this is so the view that the cold agglutinins in sera 1 and 5 are a combination of the specific and non-specific varieties is not tenable. The fact that in certain sera (Bird, 1951*a*) the separate specific nature of auto-agglutinin

TABLE III.—*Cold Agglutinin Titres Before and After Absorption with O Cells at 4° and Activity of Eluted Antibody.*

Sample.	Group.	Predominant agglutinins.	Titre before absorption with appropriate O cells.*					Titre after absorption with appropriate O cells.				Activity of agglutinin solution.			
			Temp.	O.	A_1.	B.	Own.	O.	A_1.	B.	Own.	O.	A_1.	B.	Own.
1	A_2B	Anti-A_1	4°	8	128	8	8	0	64	2	4	W	+	W	W
			37°	0	8	0	0	0	2	0	0	—	W	—	—
2	A_1 Le (a−b−)	Anti Le^a and anti Le^b	4°	16	16	..	16	0	4	..	8	—	—	..	—
			37°	2	2	..	0	0	0	..	0	—	—	..	—
3	A_1 Le (a−b−)	Anti Le^a and anti Le^b	4°	16	16	..	8	0	4	..	2	—	—	..	—
			37°	1	1	..	0	0	0	..	0	—	—	..	—
4	B Le (a−b+)	Anti-Le^a	4°	16	..	16	1	2	..	2	0	W	—	W	—
			37°	1	..	1	0	0	..	0	0	—	—	—	—
5	A_2B	Anti-A_1	4°	8	256	8	4	0	16	0	0	W	+	W	..
			37°	0	16	0	0	0	4	0	0	—	W	—	—
6	A_1B	Anti-O	4°	16	2	2	2	0	32	32	16	W	—	—	—
			37°	1	0	0	0	0	0	0	0	—	—	—	—

* Sera 2 and 3 were absorbed with a sample of O Le (a−b−) cells. After absorption they were titrated with O Le (a−b+) cells. Serum 4 was absorbed with O Le (a−) cells. After absorption it was titrated with O Le (a+b−) cells.

TABLE IV.—*Iso-Agglutinin Titres of Various Sera Before and After Absorption with O Cells in the Cold.*

Sample number.	Blood group.	Titre before absorption.		Titre after absorption.	
		Anti-A.	Anti-B.	Anti-A.	Anti-B.
1	A	—	64	—	32
2	O	64	32	64	16
3	A	—	16	—	8
4	B	32	—	16	—
5	O	32	16	16	8
6	A	64	—	16	—
7	A	—	8	—	4
8	A	—	8	—	8
9	A	—	16	—	8
10	A	—	128	—	64
11	A	—	32	—	16
12	A	—	16	—	16
13	B	32	—	32	—
14	B	64	—	32	—
15	B	64	—	64	—
16	B	32	—	16	—
17	A	—	32	—	32
18	B	64	—	32	—
19	A	—	16	—	16
20	A	—	64	—	32
21	A	—	16	—	16
22	A	—	16	—	16
23	O	32	32	32	16
24	B	128	—	64	—
25	B	32	—	32	—
26	O	256	16	128	16
27	O	512	128	256	64
28	A	64	—	32	—
29	B	16	—	16	—
30	B	128	—	64	—
31	B	32	—	16	—
32	A	—	64	—	64

components can be demonstrated suggests that linkage is the more probable explanation; that is, that auto-agglutinins in most sera may possess multiple combining sites and so render the various component antibodies undemonstrable. The evidence presented is suggestive but not conclusive.

The enhancement of activity against A and B cells in serum 6 (Table IV) after absorption with O cells may be of importance in providing a pointer to the structure of auto-agglutinins. Similar sera have been described by Bird (1951*b*). It is possible that during antibody formation certain receptors are laid down first, and more superficial receptors are then formed around these. It would then be easy to understand how, in certain sera, the removal of the anti-O receptors by

TABLE V.—*Agglutinin Titres of Serum* 3.

1. Before neutralisation :

	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
A cells	—	—	—	+	+	+	+	—	—
B cells	—	—	+	+	+	+	+	—	—

2. After absorption with A substance :

A cells	—	—	—	—	—	—	—	—	—
B cells	+	+	+	+	+	+	+	—	—

3. After absorption with B substance :

A cells	+	+	+	+	+	+	+	—	—
B cells	—	—	—	—	—	—	—	—	—

absorption would expose the deeper receptors. This concept has been briefly propounded by Bird (1952).

SUMMARY.

Anti-A——anti-B linkage (dual-receptor antibody structure) occurs in human sera. It is suggested that similar linkage (multi-receptor antibody structure) often occurs in the case of auto-agglutinins and renders the various component antibodies inseparable by absorption.

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THE DEPOSITION OF ^{91}Y IN RABBIT BONES.

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KNOWLEDGE of the metabolism of yttrium is urgently required because radioactive yttrium isotopes are themselves important fission products, and also one, ^{90}Y, is produced by the radioactive decay of ^{90}Sr. It is known that the greater part of any yttrium reaching the blood stream is concentrated in the skeleton, and that the deposition of tracer doses of radioactive yttrium in bone differs both in localization and mechanism from that of certain other bone-seeking elements, notably strontium and phosphorus (Vaughan, Kidman and Tutt, 1952; Macdonald, Nusbaum, Alexander, Ezmerlian, Spain and Round, 1952). The present paper describes observations on the distribution of ^{91}Y in the skeleton of rabbits of different ages at varying periods after a single intravenous injection.

EXPERIMENTAL

Rabbits of the same stock as those used in previous experiments (Kidman, Tutt and Vaughan, 1950; Tutt, Kidman, Rayner and Vaughan, 1952) were fed on a diet of oats, hay and cabbage. They were given a single intravenous injection of ^{91}Y as YCl_3 in doses varying from 12 to 500 μc. according to the length of the experiment. Only traces of carrier were present in the solution, which was kept acid (pH 1–3) to prevent adsorption on glass or formation of colloids. Three groups of animals of different ages were used:

(i) Of 18 rabbits aged 5–7 weeks 3 were killed 10 minutes, 24 hours, 9 days, 8 weeks, 16 weeks and 24 weeks after injection. The urinary and faecal excretion of ^{91}Y was estimated for 5 days before the animals in the last group were killed.

(ii) Twelve rabbits aged 3–4 months were killed in groups of 3, 10 minutes, 24 hours, 9 days and 21 days after injection.

(iii) Two rabbits a year old were killed 10 minutes and 9 days after injection respectively.

Immediately after death the bones were removed as previously described. (Kidman, Rayner, Tutt and Vaughan, 1952; Tutt *et al.*, 1952). The right femur and usually the right tibia were used for the preparation of autoradiographs; one or both tibia-fibula, and both humeri, were used for the determination of yttrium retention in different parts of the bone in animals aged 5–7 weeks and 3–4 months. The bones were divided into epiphysis, epiphyseal plate, metaphysis and diaphysis in animals killed at intervals up to 8 weeks after injection. After that time they were divided into "ends" and diaphysis only. Figures for the tibia-fibula only are given; those for the humerus followed the same pattern. The yttrium content of the scapula, pelvic girdle, radius and ulna and left femur was determined separately, and that for the "rest of the bone" together since it was difficult to clean and therefore to weigh the bones with any accuracy. In order to obtain a figure for the total percentage of injected yttrium in the skeleton, the figure obtained for the left femur was doubled and a calculated figure added to represent the tibia and humerus content. The amount of isotope present is expressed throughout as a percentage of the injected dose, and the weight of bone is also given, since in younger animals the growth dilution factor is considerable. It must be remembered, however, in interpreting the Tables that although the percentage retained in

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any two bones may be the same, the concentration of isotope may be different. The percentage retained is related to the initial uptake, but the growth dilution factor varies from bone to bone. A statistical analysis of the results was carried out, taking into consideration the interaction of percentage retention and gain in weight.

Autoradiographs.

Autoradiographs were prepared from sections of bone as previously described (Kidman *et al.*, 1952), all sections being embedded in perspex prepared from methyl methacrylate monomer. Exposure was regulated to give a satisfactory macroscopic picture. Degrees of blackening in different autoradiographs cannot be compared, although differences in different parts of one autoradiograph are significant. Sections of normal bone containing no radioactive isotope were exposed as controls and were always negative.

Estimation of ^{91}Y.

Estimation of ^{91}Y in the bones was carried out as described for similar experiments with radioactive strontium. (Kidman *et al.*, 1950 ; Tutt *et al.*, 1952).

RESULTS.

Autoradiographs.

Autoradiographs prepared from the long bones showed two types of reaction, localized and diffuse. The picture was unaffected by the size of the dose within the range given.

Localized reactions were found in young animals in the following sites immediately after injection : (*i*) a strong reaction in the region of the lower edge of the epiphyseal plate which extended down less strongly into the metaphyseal trabeculae ; (*ii*) a narrow band above the epiphyseal and beneath the articular cartilage ; (*iii*) a fine line outlining bony trabeculae and the whole of the periosteal and endosteal surface of the bone which was possibly more intense on the periosteal surface of the metaphysis ; (*iv*) irregular streaks and dots throughout the diaphysis (Fig. 1 and 7). In the weanling rabbits 9 days after injection there was wide separation of the two bands adjacent to the epiphyseal plate, the lower one appearing only as a series of dots on the lower end of the metaphyseal trabeculae. The other localized reactions appeared unaltered (Fig. 2). Eight and 16 weeks after injection in the young rabbits there was still a heavy localized reaction in the bony trabeculae in the centre of the epiphysis. The speckling in the shaft was still present, and at varying points dependent on the rate of growth of the bone there was an area of strong reaction at some distance from the epiphysis in the diaphysis. This point appeared to correspond to the lower end of the metaphysis at the time of injection, and is presumably due to incorporation of the most lateral trabeculae in the diaphysis (Fig. 3). The periosteal and endosteal lines were less clearly defined. The picture was unchanged 6 months after injection (Fig. 4). Changes of the same order but less marked were seen in autoradiographs from the bones of rabbits 3–4 months old (Fig. 5). Bones from animals a year old immediately after injection showed a clear outlining of all bony trabeculae, of periosteal and endosteal surfaces and speckling throughout the diaphysis and all bony trabeculae (Fig. 6).

Diffuse reaction.—At all ages there was a faint diffuse reaction throughout mineralized bone immediately after injection. This diffuse reaction was still present six months after injection in all bone which was formed before the injection. It was also present in the diaphysis on the metaphyseal side of the localized

points already described extending with decreasing intensity towards the epiphysis. A diffuse reaction in the metaphyseal trabeculae was present but visible only microscopically. A more definite diffuse reaction was apparent in spongy bone external to that showing a localized reaction in the epiphysis.

Chemical Analysis.

(a) *5–7-week-old rabbits.*

Total retention in the skeleton 10 minutes after injection, expressed as percentage of the injected dose, was 43·8 ± 4·3, after 24 hours it was 75·0 ± 2·3, after 9 days 75·8 ± 0·5 ; it then fell extremely slowly, being still 59·5 ± 3·2 six months after the injection (Table I). The average daily excretion in the urine six months after injection was 0·07 per cent, and in the faeces 0·02 per cent of the injected dose.

TABLE I.—*Mean Values for* ^{91}Y *(Expressed as Percentage of the Injected Dose) Retained in the Skeleton of Rabbits Killed at Varying Time Intervals after Injection (3 Rabbits in each Group).*

Time after injection.	*Age at time of injection.*	
	5–7 weeks.	3–4 months.
10 minutes	43·8±4·3*	30·3±0·7
24 hours	75·0±2·3	53·2±4·0
9 days	75·8±0·5	71·7±7·9
21 ,,	—	66·8±2·4
8 weeks	70·0±1·4	
16 ,,	65·1±4·3	
24 ,,	59·5±3·2	

* S.E. $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$ · x = individual observation. $\bar{x}$ = means of observations. n = number of observations in group.

DESCRIPTION OF PLATES.

Autoradiographs prepared from sections of lower end of femur of rabbits 5–7 weeks old at time of injection with ^{91}Y. (*All* × 3.)

FIG. 1.—10 minutes after injection (100 μc./kg.). Note (*a*) intense localized reaction beneath the epiphyseal plate ; (*b*) speckled reaction in the diaphysis ; (*c*) narrow periosteal and endosteal lines.

FIG. 2.—9 days after injection (100 μc./kg.). Note (*a*) intense localized reaction is now at extreme lower end of metaphyseal trabeculae.

FIG. 3.—8 weeks after injection (200 μc./kg.). Note (*a*) loss of reaction in the metaphysis : (*b*) two localized areas of reaction in diaphysis presumed to be remains of lateral metaphyseal trabeculae at time of injection incorporated in the diaphysis ; (*c*) persistence of localized reaction in centre of epiphysis ; (*d*) faint reaction in diaphysis in areas of new bone formation.

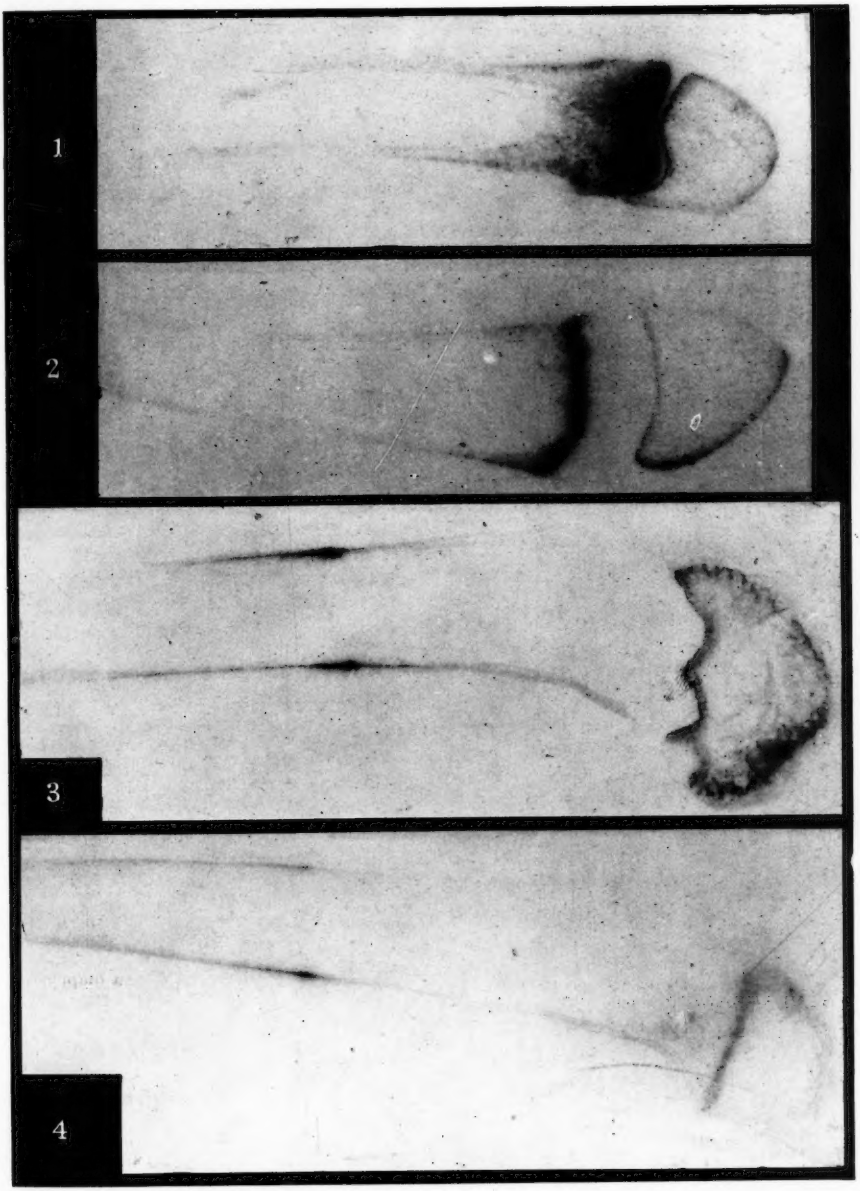
FIG. 4.—24 weeks after injection (500 μc./kg.). Note diffuse reaction throughout diaphysis and epiphysis.

Autoradiographs prepared from sections of lower end of femur of older rabbits after an injection of ^{91}Y.

FIG. 5.—3–4-month-old rabbit killed 21 days after injection (180 μc./kg.). Note (*a*) persistence of localized reaction in the metaphysis ; (*b*) outlining of periosteum and endosteum. (× 3.)

FIG. 6.—12-month-old rabbit killed 10 minutes after injection (100 μc./kg.). Note (*a*) outlining of trabeculae and of periosteal and endosteal surface of bone ; (*b*) speckling in shaft and trabeculae. (× 3.)

FIG. 7.—High-power view of autoradiograph of cortical bone, showing localized reaction giving speckling against a background of diffuse reaction. (× 100.)



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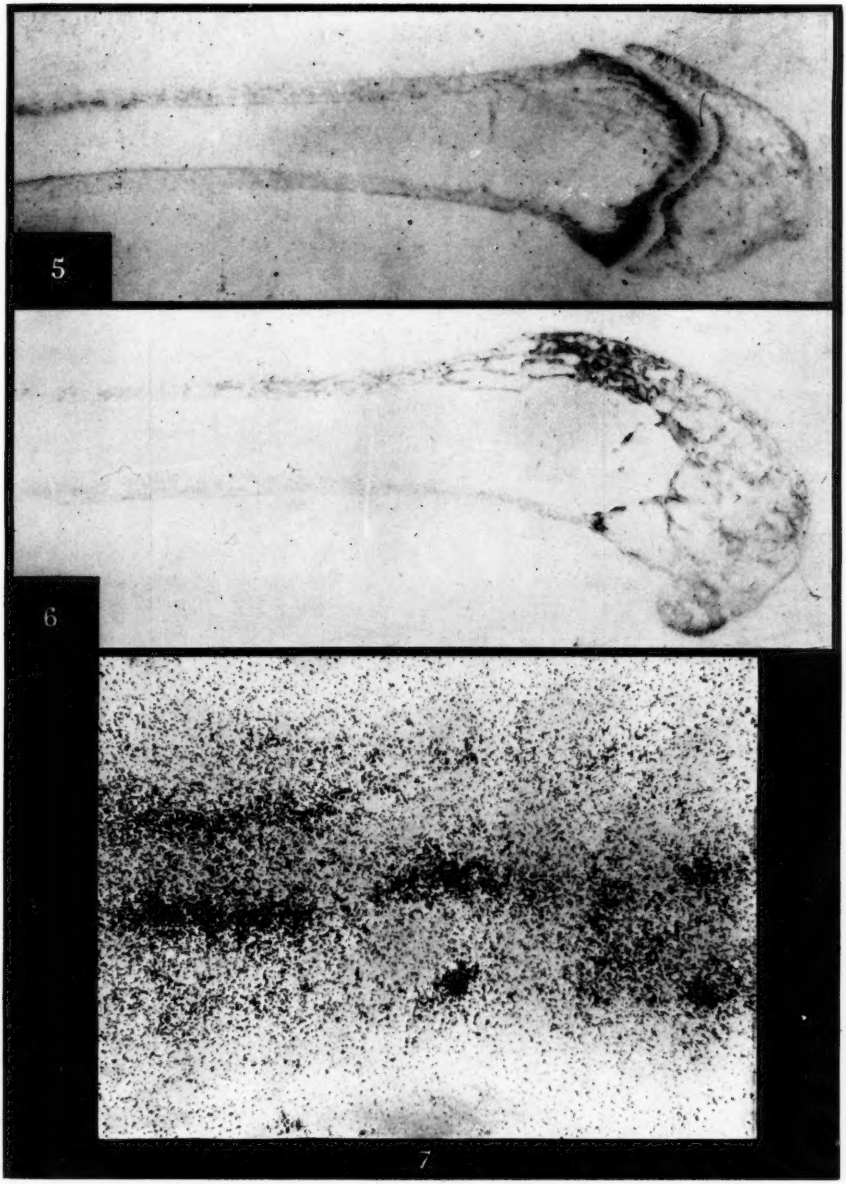

Rayner, Tutt and Vaughan.

TABLE II.—*Weights of Bones and Percentages of Injected ^{91}Y Retained at Different Times after Injection in 5–7-Week-old Rabbits. (Mean of 3 Bones at each Point).*

Time after injection.	Femur.		Radius-Ulna.		Scapula.		Pelvic Girdle.		"Rest of Bone."	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	4·45±0·18	4·51±0·44	1·73±0·12	1·58±0·14	1·18±0·14	1·16±0·02	2·63±0·10	2·89±0·18	41·0±1·5	27·55±3·42
24 hours	5·47±0·27	10·04±0·61	2·23±0·22	3·81±0·21	1·50± —	2·52±0·10	3·30±0·15	6·25±0·06	47·3±2·3	38·58±0·82
9 days	6·77±0·20	8·84±0·23	2·60±0·06	2·72±0·11	1·95±0·17	2·07±0·09	4·48±0·07	5·47±0·22	57·7±4·1	45·15±1·00
8 weeks	10·37±0·44	7·43±0·21	3·36±0·13	2·45±0·11	3·18±0·19	1·94±0·06	7·19±0·83	4·85±0·20	92·3±3·9	43·50±1·42
16 „	12·67±0·67	6·40±0·33	4·00± —	2·78±0·56	3·50±0·29	1·70±0·08	8·50±0·29	3·38±0·63	105·0±2·6	41·64±3·47
24 „	11·83±0·44	6·43±0·18	4·50± —	2·36±0·07	4·67±0·44	1·62±0·03	8·83±0·17	3·99±0·12	113·7±1·8	36·30±2·85

TABLE III.—*Weights of Portions of Tibia-fibulas and Percentages of ^{91}Y Retained at Different Times after Injection in 5–7-Week-old Rabbits.*

Time after injection.	Epiphysis.		Epiphyseal Plate.		Metaphysis.		Diaphysis.	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	0·162±0·007	0·39±0·01	0·144±0·015	0·64±0·04	0·220±0·013	0·59±0·13	0·298±0·012	0·33±0·02
24 hours	0·167±0·011	0·58±0·05	0·177±0·013	1·79±0·06	0·251±0·012	1·21±0·08	0·370±0·013	0·49±0·02
9 days	0·261±0·027	0·55±0·05	0·246±0·011	0·64±0·03	0·374±0·022	1·57±0·20	0·557±0·031	0·71±0·06
8 weeks	0·323±0·004	0·53± —	0·344±0·009	0·46±0·01	0·463±0·054	0·52±0·04	1·097±0·084	1·32±0·08

TABLE V.—*Weights of Bones and Percentages of Injected ^{91}Y Retained at Different Times after Injection in 3–4-Month-old Rabbits. (Mean of 3 Bones at Each Point).*

Time after injection.	Femur.		Radius-Ulna.		Scapula.		Pelvic Girdle.		"Rest of Bone."	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	11·8±0·2	3·29±0·24	4·3±0·3	1·36±0·04	3·7±0·2	0·97±0·05	8·3±0·3	2·16±0·10	95·3±2·4	17·82±1·04
24 hours	10·7±0·9	6·80±0·92	3·7±0·2	2·21±0·17	3·7±0·3	1·70±0·06	8·0±0·6	4·15±0·33	87·7±3·4	29·36±1·56
9 days	11·7±0·7	8·52±1·33	4·5±0·3	3·01±0·22	3·8±0·3	2·33±0·26	8·5±0·8	5·93±0·61	97·3±4·3	40·31±4·00
21 „	13·3±0·3	7·05±0·39	5·0±0·8	2·75±0·31	4·3±0·4	2·34±0·12	9·5±0·5	4·90±0·32	110·0±5·0	41·19±1·54

TABLE VI.—*Weights of Portions of Tibia-fibulas and Percentages of ^{91}Y Retained at Different Times after Injection in 3–4-Month-old Rabbits.*

Time after injection.	Epiphysis.		Epiphyseal Plate.		Metaphysis.		Diaphysis.	
	Weight (g.).	Per cent.	Weight (g).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	0·348±0·033	0·16±0·02	0·373±0·022	0·58±0·11	0·580±0·088	0·42±0·52	1·294±0·155	0·40±0·07
24 hours	0·319±0·331	0·29±0·04	0·352±0·020	1·18±0·09	0·483±0·034	0·82±0·07	1·182±0·081	0·73±0·07
9 days	0·351±0·045	0·56±0·20	0·391±0·014	1·06±0·17	0·512±0·070	1·14±0·22	1·264±0·078	0·90±0·09
21 „	0·330±0·021	0·27±0·03	0·479±0·021	0·68±0·10	0·556±0·037	0·70±0·08	1·661±0·109	0·83±0·08

Different bones.—The percentage of the injected dose retained in different bones directly after injection was roughly proportional to their initial weight (Table II). Uptake in the femur, radius, ulna, pelvic girdle and scapula reached its maximum 24 hours after injection, while in the " rest of bone " the highest figure was found 9 days after injection. There was then a slow fall in all bones except for an apparent rise at six months in the pelvic girdle. Unless such a result is confirmed on repetition its significance is doubtful. With this exception the downward trend may be regarded as significant, since although any particular difference observed may not be significant, the accumulation of several may ; thus for the femur, although neither the decrease from 24 hours to 9 days nor that from 9 days to 8 weeks is significant, the total decrease from 24 hours to 8 weeks is significant, showing that the apparent downward trend is genuine. The pattern of retention is then the same in the long and flat bones examined separately. In one or more of the bones included in " rest of bone " this pattern would appear to be different.

Different parts of the same bone.—Chemical analysis of retention in different parts of the bone showed that in all parts there was an increase in the percentage retained between 10 minutes and 24 hours after injection. Thereafter the percentage in the epiphysis was unchanged, that in the diaphysis increased, and that in the epiphyseal plate and metaphysis decreased up to 8 weeks after the injection (Table III). These differences were highly significant ; thus the percentages found 24 hours and 8 weeks after the injection were 0·49 and 1·32 respectively in the diaphysis, and 1·79 and 0·46 respectively in the epiphyseal plate. Analysis of " ends " and diaphysis six months after injection showed the diaphysis still contained 1·34 per cent of the injected dose, while the " ends " contained 1·02 per cent compared with 3·57 per cent at 24 hours (Table IV).

TABLE IV.—*Weights of " Ends " and Diaphysis of Tibia-fibulas and Percentages of* ^{91}Y *Retained at Different Times after Injection in 5–7-Week-old Rabbits.*

Time after injection.	" Ends."		Diaphysis.	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes . . .	0·526±0·016	1·62±0·10 .	0·298±0·012	0·33±0·02
24 hours . . .	0·596±0·033	3·57±0·12 .	0·370±0·013	0·49±0·02
9 days . . .	0·881±0·042	2·76±0·26 .	0·557±0·031	0·71±0·06
8 weeks . . .	1·130±0·058	1·52±0·26 .	1·097±0·084	1·32±0·08
16 „ . . .	1·236±0·059	1·12±0·06 .	1·618±0·166	1·21±0·13
24 „ . . .	1·329±0·034	1·02±0·03 .	1·763±0·052	1·34±0·04

(b) 3–4-*month-old rabbits.*

Total retention in the skeleton expressed as percentage of the injected dose was 30·3 ± 0·7 10 minutes after injection, 53·2 ± 4·0 after 24 hours, 71·7 ± 7·9 after 9 days, and 66·8 ± 2·4 after 21 days (Table I).

Different bones.—The maximum retention in all bones was found 9 days after injection. Except in the femur, the increase between 24 hours and 9 days was significant. Twenty-one days after injection there was no significant decrease. The pattern of retention was the same both in the long and flat bones examined separately and in the " rest of bone " (Table V).

Different parts of the same bone.—Analysis of the retention in different parts of the bone shows that the differences at different times, although not as dramatic

as in younger animals, were significant at certain time intervals (Table VI). Thus there was a rise in the percentage of yttrium in all parts of the bone between 10 minutes and 24 hours after the injection. The epiphysis showed a further rise from 0·29 per cent 24 hours after injection to 0·56 per cent at 9 days, followed by a fall to 0·27 per cent at 8 weeks. The percentage in the plate decreased significantly from 1·18 at 24 hours to 0·68 at 9 days ; that in the metaphysis showed a rise from 0·82 at 24 hours to 1·14 at 9 days, and then a fall to 0·70 at 21 days. The percentage in the diaphysis did not rise or fall significantly between 24 hours and 8 weeks after the injection, the value remaining between 0·73 and 0·83.

DISCUSSION.

The chemical and autoradiographic results recorded here confirm previous observations (Vaughan *et al.*, 1952 ; Kidman, Tutt and Vaughan, 1951) that the uptake of radioactive yttrium in tracer doses by the rabbit's skeleton is extremely rapid at all ages, A rather higher percentage of the yttrium injected is taken up than of radioactive strontium, although if given in other than tracer amounts bone appears more avid for strontium (Macdonald *et al.*, 1952). The yttrium appears to be retained longer than the strontium, and to be less affected in its uptake by age. The higher uptake affects all parts of the bone in young animals. The fact that excretion of ^{91}Y in urine and faeces is lower at both 9 days and at 6 months (Kidman *et al.*, 1951) after injection than that of ^{90}Sr is compatible with this higher retention.

The significant point which arises from analysis of the yttrium content in different whole bones is that in the 5–7-week-old group the rate of uptake in the " rest of bone " is slower than in the separate bones examined. This effect is not apparent in the 3–4-month-old group. It is not yet known whether all or part only of the bones included in " rest of bone " are responsible for this phenomenon, nor whether it is the yttrium lost from the other bones or from the soft tissues between 24 hours and 9 days after injection that is taken up by the " rest of bones."

The autoradiographic studies suggest that as in the case of strontium (Kidman *et al.*, 1952) and phosphorus (Leblond, Wilkinson, Belanger and Robichon, 1950) ^{91}Y given in a tracer dose is deposited in bone in two ways, giving respectively a heavy localized reaction and a diffuse reaction. The latter may well be due to adsorption on bone salt or protein surfaces (Tutt *et al.*, 1952 ; Kidman *et al.*, 1952). Bone formed after injection, both in the shaft and in the epiphysis, also shows a diffuse reaction. As the length of new bone formed is considerable in some cases, this is presumably due to uptake from the blood stream of ^{91}Y released in the normal process of resorption or from the soft tissues. This secondary uptake has been observed in 24-hour-old rabbits following a single injection of ^{90}Sr, but is much less definite in 5–7-week-old rabbits given ^{90}Sr (personal observations, unpublished). Presumably the element in bone that takes up ^{91}Y takes it up more readily and when in a lower concentration in the blood than that which takes up ^{90}Sr. This secondary uptake together with considerable retention in the epiphysis may to a large extent account for the maintenance of a high total retention in the skeleton after resorption of the metaphyseal trabeculae containing the isotope laid down at the time of injection. If yttrium were to form a complex with some component of the connective tissue of bone as has been suggested (Hamilton, 1947 ; Copp, Hamilton, Jones, Thompson and Cremer, 1952), this

may account for its continued retention, since the metabolic turnover of bone collagen is extremely slow (Perrone and Slack, 1951). It is, however, necessary to postulate a difference in the connective tissue in different sites, since yttrium is taken up in concentration by rapidly growing bone tissue beneath the epiphyseal plate giving a strong localized reaction, but not by active bone tissue beneath the endosteum and periosteum (Fell, 1931–32). The narrow line along the entire periosteal and endosteal surfaces of bone immediately following injection is possibly due to deposition of ^{91}Y in periosteum and endosteum, while the speckling in mineralised bone may depend on deposition in some element of connective tissue around Haversian systems in a manner similar to that following the injection of americium (Scott, Axelrod, Fisher, Crowley and Hamilton, 1948) and curium (Scott, Axelrod and Hamilton, 1949). That only certain systems are affected may be due to the fact that in bones as in other organs only a proportion of the vessels is patent at any one time, and the isotope is taken up in relation to those vessels which are patent when the blood level is high.

SUMMARY.

Chemical and autoradiographic studies show that the deposition of ^{91}Y in the bones of rabbits of different ages following a single intravenous injection of a carrier-free tracer dose is extremely rapid. Retention is greater, rather more persistent and less affected by age than that of strontium. Excretion in both urine and faeces is rather less than that of strontium.

^{91}Y appears to be concentrated (*i*) in the site of active bone growth beneath the epiphysis but not beneath the endosteum or periosteum, suggesting differences in the character of the connective tissue of bone in the two sites ; (*ii*) in a patchy way in the shaft presumably in association with the connective tissue around blood-vessels.

New bone in young animals formed months after the original injection contains traces of ^{91}Y, presumably due to the capacity of some element in bone to complex with ^{91}Y present in extremely low levels in the blood as a result of normal processes of resorption.

We should like to express our thanks to Professor J. H. Burn, whose constant encouragement has made this work possible, and to Dr. M. R. Sampford for his statistical analysis of our results. One of us (J. V.) has had a personal grant from the Medical Research Council. We are also indebted to the Haematology Research Fund endowed by the Nuffield Foundation.

This work was begun on behalf of the Protection Sub-Committee of the Medical Research Council's Committee on Medical and Biological Applications of Nuclear Physics.

A copy of the statistical analysis can be obtained on request. The conclusions are embodied in the text.

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THE GOITROGENIC ACTION OF RESORCINOL IN RATS.

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THE antithyroid action of resorcinol (*meta*-dihydroxyphenol) was demonstrated by Bull and Fraser (1950) in 3 patients suffering from myxoedema and goitre induced by the prolonged application of resorcinol ointment to varicose ulcers. Hart and Maclagan (1951) and Marsh (1951) recently reported 2 further cases. Doniach and Fraser (1950) found that subcutaneous injection of a watery solution of resorcinol in rats lowered their thyroid iodine uptake over a 2-hr. period by inhibition of organic binding of iodide in a manner comparable with that produced by methylthiouracil. This led Arnott and Doniach (1952) to test the antithyroid potency of a series of 35 allied compounds. Derivatives of resorcinol itself, including resorcinol diacetate, proved strongly antithyroid whereas the *para* (quinol) and *ortho* (catechol) isomers and their derivatives were not active.

There has so far been no confirmation of the goitrogenic action of resorcinol in experimental animals. In fact there are two reports of failure. Klein, Ottis and Velvart (1950) injected 50 mg. resorcinol per kg. body wt. daily as a 1 per cent watery solution into rabbits for 15 days, and Cheymol, Gay and Lavedan (1951) injected the same dose every 48 hr. into mice for 1 month. No thyroid hyperplasia was obtained in the rabbits or the mice.

The object of this paper is to describe our attempts to establish the goitrogenic action of resorcinol in rats. In view of the rapid metabolism and excretion of the dihydric phenols (Garton and Williams, 1949), we sought for modes of application which might be expected to maintain an effective concentration of the drug in the blood. It was clear that the injection of an aqueous solution once a day did not do this. We thought that a slower release into the circulation of small quantities of resorcinol might be effected by percutaneous inunction or by subcutaneous injection of an oily solution or suspension. We also considered the possibility of obtaining a slow release of resorcinol from the hydrolysis of its diacetate.

MATERIAL.

Rats.—Albinos of mixed origin, of both sexes, weighing 100–300 g.

Resorcinol ointment (12·5 *per cent*).—Resorcinol 12·5 g., glycerin 12·5 g., wool fat 37·5 g., yellow soft paraffin 37·5 g.

Resorcinol in oil.—Resorcinol 1·5 g. in 100 ml. arachis oil.

Resorcinol in beeswax and oil.—Resorcinol 10 g. in 100 ml. arachis oil containing 5 g. beeswax.

Resorcinol diacetate, quinol diacetate, catechol diacetate.

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EXPERIMENTAL.

Application of resorcinol ointment.

The shaved bellies of 6 rats were rubbed for ¼ hr. twice daily over a period of 3 weeks with resorcinol ointment; 6 controls were similarly treated with the ointment base only. No difference was observed at the end of the experiment between the weights of the thyroids of the two groups.

Injection of resorcinol in oil.

Resorcinol in oil (0·14 m mol./100 g. body wt.) was injected subcutaneously into 4 rats in 2 ml. amounts twice daily, while 3 control rats received the oil only. The resorcinol-treated rats were killed after time intervals of 10, 31, 47 and 69 days of treatment. At 47 days 1 control rat was killed and the remaining 2 at 69 days. The thyroid wt. of the controls were 16, 18 and 13 mg. and of the 47- and 69-day resorcinol-treated rats 32 and 25 mg. respectively. The control thyroids showed a normal histology. The 32-mg. gland showed the typical goitrogen-induced changes of hyperaemia, gross cellular hyperplasia and widespread depletion of colloid. The 25-mg. gland showed a moderate hyperplasia. The thyroids of the resorcinol-treated rats killed at 10 and 31 days appeared macroscopically normal and were unfortunately discarded.

These findings demonstrate the goitrogenic action of frequent injections of resorcinol in oil. The method was not pursued, since the increasingly large oil depots which collected in the rats' flanks were presumed to have led to an irregular absorption of the drug.

Injection of resorcinol in beeswax and oil mixture.

Twice daily subcutaneous injection of 0·5 ml. of warm resorcinol in beeswax-arachis-oil mixture (0·45 m mol./100 g. body wt.) was given to 6 rats. An injection of the beeswax-arachis-oil alone was given to 3 controls. The concentration of resorcinol absorbed from this mixture proved toxic, produced convulsions, and killed the rats after only a few days' treatment.

The persistence of antithyroid activity after administration of various doses of resorcinol diacetate.

Preliminary tests showed that resorcinol diacetate, which is a liquid at room temp. was non-toxic when injected neat in doses of up to 1·0 m mol./100 g., in contrast to resorcinol in water, which produced convulsive tremors when given at 1/10th of this concentration. An attempt was made to find out how long antithyroid activity persists after various doses. The method of assay described by Arnott and Doniach (1952) was used. The thyroid uptake of radioactive iodine of treated rats was compared with that of controls. The results given in Table I show that one injection of 0·4 m mol. diacetate/100 g. maintains an antithyroid action for at least 9 hr. and 1·0 m mol./100 g. for 19 hr. The former reduced the iodine uptake by the thyroid to 15·2 per cent of the controls during the period of 7 to 9 hr. after the drug, and the latter to 19·8 per cent during the period of 17 to 19 hr. after the drug.

TABLE I.—*The Effect of a Single Injection of Resorcinol Diacetate on the Thyroid Uptake of Radioactive Iodine over a 2-Hour Period in Rats some Hours after Administration of the Drug.*

	Treatment.	Rat weight (g.).	Time (hr.)— Between injection of compound and of ^{131}I.	Time (hr.)— Between injection of ^{131}I and killing.	Count rate per minute.	Mean + standard error of mean.	Thyroid uptake of ^{131}I (as per cent of controls).
A	0·2 m mol./100 g.	180	17	2	7189	7200±500	113
		190			6614		
		182			8687		
		175			7901		
		190			5409		
B	1·0 m mol./100 g.	190	17	2	1064	1256±224	19·8
		190			882		
		210			1349		
		180			739		
		180			2250		
	Controls for A and B	200	—	2	5515	6353±263	100
		160			6704		
		200			5951		
		220			7218		
		190			6377		
C	0·4 m mol./100 g.	190	7	2	3146	2019±310	15·2
		190			1556		
		198			1385		
		198			2253		
		180			1805		
	Controls for C	205	—	2	17883	13290±1410	100
		193			11606		
		194			8953		
		183			14718		

The thyroids of groups A and B and their controls were assayed together 7 days after removal. Their count rates were lower than those of group C and its controls, which were assayed the day after removal, because of the natural radioactive decay of ^{131}I.

Injection of the diacetates of resorcinol, quinol and catechol.

Rats in 3 groups were injected subcutaneously twice daily, 9.30 a.m. and 6.30 p.m., with 0·4 m mol./100 g. respectively of the diacetates of resorcinol, quinol and catechol. A fourth group was similarly injected with normal saline. All were females of about 200 g. The quinol and catechol diacetates are solid at room temp., are insoluble in water, and were therefore suspended in normal saline containing 3 per cent glycerin and albumen. The same quantity of glycerin

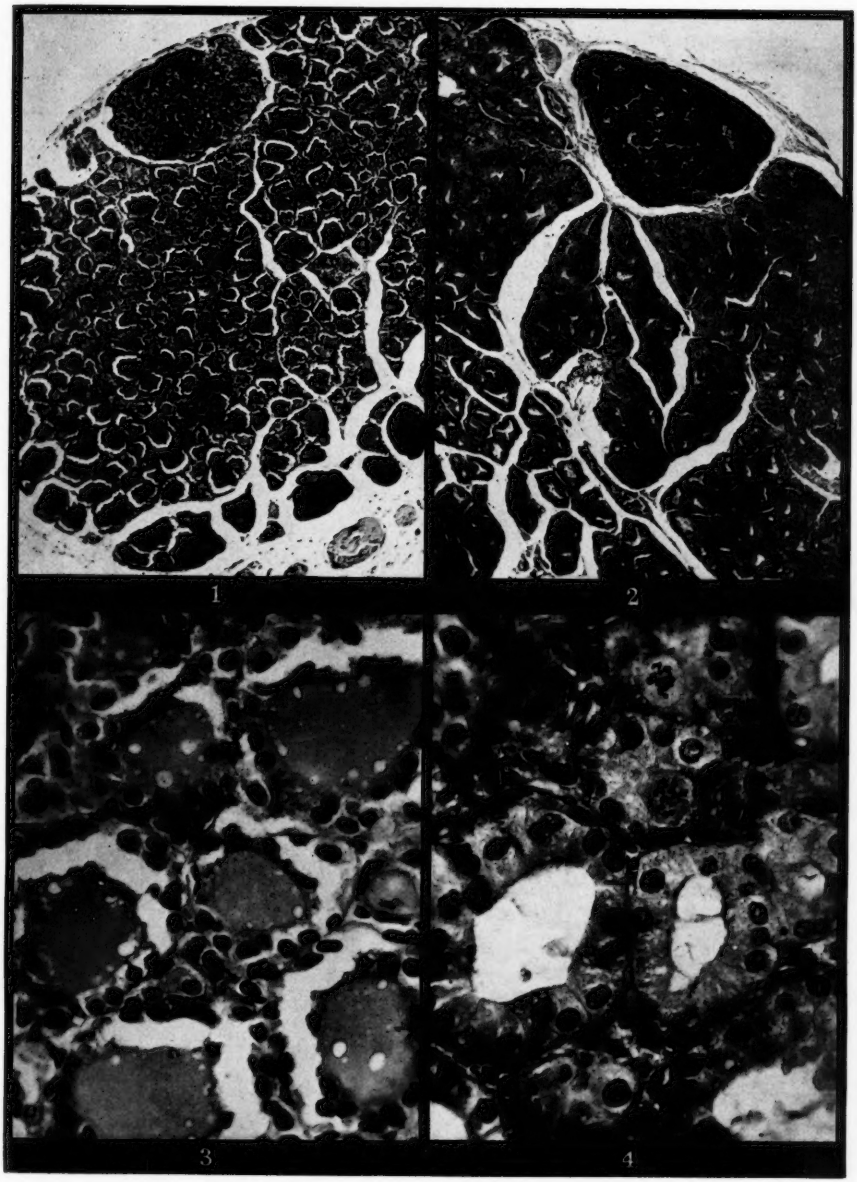
DESCRIPTION OF PLATES.

FIG. 1.—Control rat thyroid. Haemalum and eosin. × 50.

FIG. 2.—Thyroid of rat injected twice daily for 25 days with resorcinol diacetate. Haemalum and eosin. × 50. The gland is hyperplastic, hyperaemic, and shows a total depletion of colloid.

FIG. 3.—Control rat thyroid. Haemalum and eosin. × 400.

FIG. 4.—Thyroid of rat injected twice daily for 12 days with resorcinol diacetate. Haemalum and eosin. × 400. The follicular cells show a 2- to 3-fold increase in height and numerous mitoses; colloid is absent.



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and albumen was added to the resorcinol diacetate and to the saline injected into the controls.

Catechol diacetate proved toxic and was abandoned after 6 injections. The quinol diacetate-treated rats were killed after 2 weeks because they developed severe local skin reactions and appeared somewhat weak and anaemic. Several of the resorcinol diacetate-treated rats were killed at the same time for comparison. The rest were killed after a total of 4 weeks' treatment, when they appeared quite healthy. The results given in Table II show that the resorcinol diacetate pro-

TABLE II.—*Effect of Twice Daily Repeated Subcutaneous Injections of* 0·4 *m mol./* 100 *g. of Resorcinol Diacetate and of Quinol Diacetate on the Thyroid Weights of Rats.*

	Compound injected.	Rat weight (g.).	No. days injected.	Thyroid weight (mg.).	Mean thyroid weight (mg.) +S.E.	Thyroid weight as per cent of controls.
A	Resorcinol diacetate	165	9	19	34·1±3·59	139
		192	9	32·5		
		200	12	40		
		195	12	40·5		
		185	12	38·5		
B	Quinol diacetate	172	9	20	21 ±1·14	86
		155	9	19·5		
		154	9	22·5		
		175	9	16		
		200	12	26		
		190	12	24		
		195	12	19		
	Saline (Controls for A and B)	222	9	25	24·5±1·24	100
		230	9	25		
		235	12	27·5		
		195	12	23·5		
		215	12	21·5		
C	Resorcinol diacetate	220	22	27	35·9±3·8	184·5
		200	25	33		
		215	25	54		
		185	25	28		
		190	25	37·5		
		205	25	36		
	Saline (Controls for C)	205	25	17·5	19·5±1	100
		210	25	23		
		210	25	21		
		190	18	17		
		218	18	19		

voked a marked thyroid enlargement after 12 days (139 per cent of controls' wt.) as well as 25 days (184·5 per cent) in contrast to the non-goitrogenic action of the quinol diacetate. Histological sections of the thyroids of the resorcinol diacetate-treated rats (12 days and 25 days) all showed increased vascularity, cellular hyperplasia and colloid depletion of varying degrees, maximal in the thyroid, which weighed 54 mg. This, together with a 12-day gland extremely rich in mitoses, is illustrated in Fig. 1, 2, 3 and 4, contrasted with a control rat's thyroid. The picture is typical of the result of thyrotrophic stimu-

lation induced by antithyroid drugs and described by Kennedy and Purves (1941), Mackenzie and Mackenzie (1943) and Astwood, Sullivan, Bissel and Tyslowitz (1943). Sections of the thyroids of the quinol diacetate-treated rats showed no hyperplasia or colloid depletion.

The non-persistence of antithyroid activity after a single injection of resorcinol in water.

Since previous workers failed to produce goitres with single daily injections of resorcinol in water, we attempted to find out in how many hours this treatment loses its antithyroid action. We know from previous work (Arnott and Doniach, 1952) that 0·05 m mol./100 g. maintains an antithyroid action for at least 2 hr.

Subcutaneous injection of 0·05 m mol./100 g. (55 mg./kg.) of resorcinol in water was given to 5 rats. Together with 5 control rats, they were each injected intraperitoneally with 10 μC of ^{131}I 3 hours later. All 10 rats were killed 2 hours afterwards, their thyroids removed and thyroid ^{131}I uptake measured. The mean count rate of the thyroids of the resorcinol-injected rats was 2884 $\pm$ 244 per min. and of the controls' thyroids 3191 $\pm$ 255 per min. The difference is not statistically significant, showing that this dose of resorcinol in water had no appreciable antithyroid potency after 3 to 5 hr.

DISCUSSION.

We wish to stress the importance in the production of thyroid hyperplasia of maintaining a continuous antithyroid level of drug in the blood stream over the 24 hours. The hyperplasia has been shown to be due to an increased output of thyrotrophic hormone by the anterior pituitary as a result of a fall in thyroxine content of the blood (Griesbach and Purves, 1943). The antithyroid drugs stop thyroxine formation. Thereafter the blood level of thyroxine is maintained only from stored hormone. This is exhausted within a few days in rats, and the greatly increased secretion of thyrotrophic hormone is perpetuated. An escape for even a few hours during the day from the antithyroid action of a drug, as occurred with the injection of resorcinol in water, owing to its rapid elimination, would be associated with temporary thyroxine formation. This thyroxine production would prevent or delay goitrogenesis in proportion to the amount of thyroxine synthesis and of available hormone already stored.

Our findings of definite goitrogenic activity show that the subcutaneous resorcinol in oil and resorcinol diacetate must have released the drug into the circulation during the hours between injections at a rate sufficient to maintain a continuous antithyroid level over the period of the experiment. In the one case the resorcinol was presumably slowly released at the oil-tissue interspace and the diacetate slowly hydrolysed in the other.

The mode of inhibition by resorcinol of organic binding of iodide in the thyroid is not known. Bull and Fraser (1950) suggested a competition with tyrosine for the iodinating mechanism. Arnott and Doniach (1952) thought it unlikely that resorcinol acted simply as a reducing agent preferentially reactive with iodine, since its isomers quinol and catechol, which are even stronger reducing agents, proved to be without antithyroid action. It is therefore of interest to observe in the above experiments that quinol diacetate proved non-goitrogenic.

SUMMARY.

By measurement of thyroid uptake of ^{131}I in rats, it was found that a subcutaneous injection of 0·05 m mol./100 g. of resorcinol in water had no antithyroid effect after 3 hr.

Subcutaneous injection of 0·4 m mol./100 g. of resorcinol diacetate maintained an antithyroid action for at least 9 hr.

Twice daily injections of resorcinol diacetate proved goitrogenic after 12 days. Quinol diacetate was not goitrogenic.

Twice daily injections of 0·14 m mol./100 g. of resorcinol in oil was goitrogenic in 1 of 2 rats after 47 days.

We are grateful to D. G. Arnott for preparing the catechol diacetate used in the above experiments and for carrying out the radioactive assays, to E. V. Willmott for the photomicrographs, J. G. Griffin and L. J. Wright for the sections, and Nadine Berger for anointing the rats with resorcinol ointment.

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THE ACTION OF TRYPSIN ON NORMAL SERUM INHIBITORS OF INFLUENZA VIRUS AGGLUTINATION.

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AT least two types of inhibitors of influenza virus agglutination have been described in normal animal sera. The first type (Francis, 1947) is heat stable (100° for 30 minutes), much more active against heated than unheated influenza B viruses, readily inactivated by the receptor destroying enzyme (RDE) of *V. cholerae* (Anderson, 1948) and is thought to be a mucopolysaccharide ; it is often known as " Francis inhibitor." A second type, present in normal rabbit serum, was described by McCrea (1946) ; it is heat labile (65° for 30 minutes), active against freshly isolated but not laboratory adapted strains of influenza virus A and is precipitated by ammonium sulphate along with gamma globulin. Heat labile inhibitors in guinea-pig, rabbit and mouse sera were later described (Ginsberg and Horsfall, 1949), and these may be similar in nature to the rabbit serum inhibitor of McCrea. However, it was Chu (1951) who first pointed out the characteristic feature of this second type of inhibitor, that it was highly active against unadapted strains of influenza virus A, but only weakly active or inactive against mouse adapted lines of the same strain ; this inhibitor may be conveniently referred to as " Chu inhibitor." Sampaio (1952) has recently studied the presence of Francis and Chu inhibitors in a number of normal animal sera. The α and β inhibitors described by Smith, Westwood and Belyavin (1951) in rabbit serum presumably correspond to Francis and Chu inhibitors.

Van der Veen and Mulder (1950) have shown that crude extracts of *V. cholerae* inactivate the inhibitor in normal ferret serum. Isaacs and Bozzo (1951) found that normal ferret serum contained mainly inhibitor of the Francis type which could be readily inactivated by purified RDE of *V. cholerae*. However, purified RDE was without effect on the Chu inhibitor present in normal rabbit serum. Chu (1951) pointed out that crude *V. cholerae* extracts, but not purified RDE, inactivate the inhibitor (Chu inhibitor) present in normal mouse serum, and Magill and Jotz (1952) and Mulder and Brans (personal communication) have found that crude *V. cholerae* extracts inactivate normal rabbit serum inhibitor.

The present investigation was begun to define more closely the action of crude *V. cholerae* extracts on Chu inhibitor. The results suggested that a trypsin-like enzyme known to be present (Stone, 1949) might be the active factor in crude *V. cholerae* extracts. It was subsequently found that crystalline trypsin was highly effective in inactivating both Francis and Chu inhibitors in all the sera examined ; the present report describes these results.

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MATERIALS AND METHODS.

Viruses.—A/England/1/51 virus was used for measuring Chu inhibitor, and Crawley (1946, England B) strain after heating at 56° for 30 min., for measuring Francis inhibitor.

Trypsin.—The preparation of trypsin used in most of these experiments was obtained from Messrs. Armour & Sons, Chicago, U.S.A. The trypsin is best dissolved in a small volume of 0·01 N HCl and then made up to the required strength by dilution in phosphate buffer, pH 8·0. Once made up in phosphate buffer the trypsin gradually loses its activity on storage at 4°. It was therefore found advisable to weigh out only small quantities at a time or to keep it in 0·01 N HCl.

Titration of non-specific inhibitor.—Serial two-fold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of 0·5 per cent suspension of fowl cells added. The same volume of virus was at once added in a dilution which gave 8 partial agglutinating doses per 0·25 ml. and the cells were allowed to settle at room temperature (20–25°). Tests were carried out in plastic plates and readings were made by the pattern method. The end-point was taken as partial (50 per cent) agglutination, and the titres in the tables are expressed as the reciprocal of the initial dilution of serum present at the end-point.

Technique of trypsin treatment.—Four parts of trypsin, made up in phosphate buffer pH 8·0 were mixed with 1 part of serum and heated at 56° for 30 min. The amounts of trypsin used are expressed in the tables as weight of crystalline trypsin per ml. of serum.

RESULTS.

Preliminary experiments were carried out to investigate the effect of crude *V. cholerae* extracts on Chu inhibitor, using normal rabbit serum as a convenient source. An extract prepared from the 4Z strain of *V. cholerae* by the method of Burnet and Stone (1947) showed only weak activity on normal rabbit serum inhibitor. Through the kindness of Professor J. Mulder and Dr. L. M. Brans, Leiden, however, a sample of crude *V. cholerae* extract prepared in their laboratory was made available to us. This preparation completely inactivated the inhibitor in normal rabbit serum and was used in the following experiments.

Action of crude V. cholerae *extracts on normal rabbit serum inhibitor.*

The technique which Mulder and Brans (personal communication) recommended for demonstrating the action of crude cholera extract was to mix four parts of extract with one of serum, incubate them together overnight at 37° and then heat for 1 hr. at 56°. In the present experiments it was found that the overnight incubation at 37° was unnecessary. Table I shows an experiment in which four parts of cholera extract were mixed with one part of serum, which was then heated at 56° for different times and titrated for inhibitory activity.

TABLE I.—*Action of Crude* V. cholerae *and Heat on Normal Rabbit Serum Inhibitor.*

Material tested.	Heated at 56° for—	Inhibitory titre.
V. cholerae extract + rabbit serum	0 min.	384
" " + " "	5 "	60
" " + " "	15 "	<12
" " + " "	30 "	<12
" " + " "	60 "	<12
Saline + rabbit serum	0 "	384
" + " "	60 "	192

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

Inhibitory activity was abolished by heating with cholera extract at 56° for 15 min. Controls showed that in the absence of heating the inhibitory activity was unaltered, while heating serum at 56° for 1 hr. in the presence of saline, phosphate buffer pH 8·0 or normal agar extract caused a two- to four-fold reduction in the inhibitory titre of rabbit serum. It appeared therefore that the combined action of a factor in crude cholera extract and the effect of heating were required for complete inactivation of inhibitor. In further experiments, therefore, the technique was modified so that the cholera extract and serum were simply mixed, heated for 30 min. at 56° and tested for inhibitory activity.

Properties of active factor in crude V. cholerae *extracts.*

Some of the properties of the active factor in crude *V. cholerae* extracts were next investigated. It was found that the active factor was adsorbed on Seitz filtration through a Ford's sterimat SB filter pad and on a 0·72 μ A.P.D. gradocol membrane. It was heat labile (Table II), and was precipitated by 50 per cent ammonium sulphate. These results suggested that the active factor might be

TABLE II.—*Effect of Heat on the Active Factor in Crude* V. cholerae *Extract.*

Material tested.	Preliminary treatment of *V. cholerae* extract.	Inhibitory titre.
Heated *V. cholerae* extract + rabbit serum	Heated 56° for 30 min.	20
" " " + " "	" 60° "	80
" " " + " "	" 62·5° "	140
" " " + " "	" 65·5° "	160
V. cholerae extract + rabbit serum	*nil*	<10
Saline + " "	"	280

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

macro-molecular, possibly a protein, and to test this possibility further the crude *V. cholerae* extract was incubated for 24 hr. at 37° with crystalline trypsin in a concentration of 8 mg./ml. of extract. The trypsin had apparently no effect on the cholera factor. However, controls showed that trypsin itself completely abolished the inhibitory action of normal rabbit serum. As with the cholera extract, it was simply necessary to mix trypsin and normal rabbit serum and heat at 56° for 30 min. in order to remove all inhibitory activity. An experiment which illustrates these results is shown in Table III.

TABLE III.—*Effect of Trypsin on Crude* V. cholerae *Extract.*

Material.	Treatment.	Inhibitory titre of serum.
Saline + rabbit serum	*nil*	384
" + " "	Heated 56° for 1 hour	192
V. cholerae extract + rabbit serum	" " "	<12
Trypsin treated *V. cholerae* extract + rabbit serum	" " "	<12
Trypsin + rabbit serum	" " "	<12

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

The active factor in *V. cholerae* extract shares a number of properties with trypsin. Thus, when trypsin and normal rabbit serum were mixed, heated at 56° for different times and then tested for inhibitory activity, the results, which

are shown in Table IV, were very similar to those which are shown in Table I for crude cholera extract. Controls showed that trypsin heated with serum did not agglutinate red cells. Crystalline trypsin made up in phosphate buffer

TABLE IV.—*Action of Trypsin and Heat on Normal Rabbit Serum Inhibitor.*

Material tested.	Heated at 56° for—	Inhibitory titre of serum.
Trypsin + rabbit serum	0 min.	640
" + " "	5 "	60
" + " "	15 "	15
" + " "	30 "	15
" + " "	60 "	15
Buffer + " "	0 "	640
" + " "	60 "	320

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

pH 8·0 was inactivated after heating at 56° for 30 min. However, its heat stability was increased when it was made up in an extract of normal agar, as used in the preparation of the cholera extract. One property which appeared at first to differentiate the two was that the active factor in crude *V. cholerae* extract was not so effective as trypsin when mixed with normal rabbit serum and incubated overnight at 37° without subsequent heating at 56°. However, in these conditions *V. cholerae* extract contains RDE, which causes "slipping" of red cells, an effect which might simulate the action of a serum inhibitor. It was found that when dilutions of the reagents were carried out in 2 per cent citrate-saline, which greatly reduces RDE action, this "prozone" was abolished; citrate-saline alone caused only a slight reduction in the prozone. In addition, the prozone was produced by *V. cholerae* extract, red cells and virus in the absence of serum. It is concluded, therefore, that *V. cholerae* extract is active on rabbit serum inhibitor after incubation at 37° alone (*cf.* Chu, 1951), and that the properties of the active factor in *V. cholerae* extract are similar in a number of respects to those of trypsin.

Action of trypsin on normal serum inhibitors.

The action of trypsin on Francis and Chu inhibitors in different animal sera was next investigated. Preliminary experiments showed that trypsin mixed with normal serum and heated at 56° for 30 min. reduced greatly, or abolished, both Francis and Chu inhibitors. An experiment was therefore carried out with different amounts of trypsin in order to determine its action on Francis and Chu inhibitors in different sera. Falling dilutions of trypsin were mixed with a constant amount of normal serum and heated at 56° for 30 min.; controls consisted of serum plus the diluent phosphate buffer, and serum plus saline. The sera were then tested for inhibitor against A/England/1/51 and heated Crawley viruses; these viruses virtually measure Chu and Francis inhibitors respectively. The results are shown in Table V.

Table V shows that the inhibitors in different sera vary in their sensitivity to trypsin. The control titres of Chu inhibitor were usually lower than the Francis inhibitor titres, and the former appeared to be more sensitive to trypsin treatment than the latter. The Francis inhibitor in rabbit serum was rather resistant, and 10 per cent of the control titre was present after treatment with 32 mg.

trypsin/ml. serum. With this exception trypsin treatment reduced inhibitory activity of these sera to an insignificant level.

TABLE V.—*Effect of Trypsin on Chu and Francis Inhibitors in Different Sera.*

Trypsin.	Serum.									
	Ferret.		Fowl.		Mouse.		Rabbit.		Guinea-pig	
	E.	H.C.	E.	H.C.	E.	H.C.	E.	H.C.	E.	H.C.
32 mg./ml. serum	<10	<10	<10	<10	<10	<10	<10	60	<10	10
16 ,, ,,	<10	<10	<10	<10	<10	<10	<10	80	<10	20
8 ,, ,,	<10	20	<10	10	<10	<10	<10	120	<10	320
4 ,, ,,	<10	960	<10	30	<10	<10	10	320	15	320
2 ,, ,,	<10	1280	<10	40	10	10	40	640	60	320
nil-phosphate control	30	1920	<10	240	20	15	60	320	60	480
nil-saline control	30	1920	<10	320	40	20	120	640	120	480

Table gives the inhibitory titres of different animal sera after treatment with trypsin and assayed against—

E. = 8 agglutinating doses of A/England/1/51.
H.C. = 8 agglutinating doses of Crawley virus heated at 56° for 30 min.

Action of trypsin on specific antibody.

In a number of experiments it was found that trypsin treatment caused a sharp drop in the normal inhibitor content of immune animal sera without any apparent action on specific antibody. Table VI shows the effect of treatment with 8 mg. trypsin/ml. serum on the normal inhibitor and specific antibody contents of immune ferret and fowl sera (to PR8 virus).

TABLE VI.—*Absence of Effect of Trypsin on Specific Antibody Titre.*

Serum.	Heated at 56° with—	Virus.		
		PR8.	A/England/1/51.	Heated Crawley.
PR8 immune ferret	Trypsin	5120	<10	10
	Buffer—control	5120	35	1280
PR8 immune fowl	Trypsin	960	<10	<10
	Buffer—control	960	<10	160

Table gives inhibitory titres of untreated and treated sera against 8 agglutinating doses of the viruses shown.

In other experiments it was found that following similar treatment, fowl, ferret, rabbit, guinea-pig, mouse and human sera showed no significant drop in antibody titre immediately or after 2 weeks' storage at 2°. Van der Veen and Mulder (1950) found no destructive effect of crude *V. cholerae* extract on specific antibody in human and ferret serum. We observed that after heating trypsin with normal serum at 56° for 30 min., tryptic activity was abolished, since on addition of a second volume of serum and heating again no further destruction of inhibitor took place.

Action of different preparations of trypsin.

Chu (1951) found that a semi-purified preparation of trypsin (Fairchild) caused a reduction in the inhibitory titre of normal mouse serum ; a crystalline preparation was without effect. Chu suggested that the activity was due to an

unidentified agent in the crude enzyme preparation. We have tested three different batches of crystalline trypsin prepared by Armour & Co., and one sample which was prepared from ox pancreas and kindly made available to us by our colleague Dr. J. Humphrey; all four preparations were highly active. The active factor was found to be heat-labile, to be effective after 30 min. incubation with serum at 37° or 56°, but not at 0°, and to show a greater activity after prolonged incubation (24 hr.) at 37°.

DISCUSSION.

These experiments were commenced in an attempt to identify the factor in crude *V. cholerae* extracts which inactivated the Chu inhibitor in normal sera. This factor was found to show similar properties to an active agent in a crystalline preparation of trypsin, and it is known that a trypsin-like enzyme is present in crude *V. cholerae* filtrates (Stone, 1949). A final identification of these factors has not yet been made, but it seems likely that the trypsin-like enzyme is the active factor in crude *V. cholerae* extract. There is as yet no evidence to suggest that the activity of the crystalline trypsin preparations is due to any impurity.

An obvious practical application of these results is in the removal of normal inhibitors from sera used for antigenic analysis of influenza viruses. Previously, workers have used either partially purified receptor-destroying enzyme of *V. cholerae* which acts on Francis inhibitor but not, apparently, on Chu inhibitor, or crude *V. cholerae* extract which acts on both Francis and Chu inhibitors. The former preparation is effective in treating ferret and fowl sera, which contain mainly Francis inhibitor, and it has the advantage that it can be readily standardised. The crude preparation was formerly the preparation of choice in treating rabbit, guinea-pig and mouse sera, but it was extremely difficult to standardise, and in addition, different batches of extract showed unpredictable variations in potency. The use of trypsin presents many advantages. It is a stable product which is active against Francis and Chu inhibitors under conditions where it does not affect the titre of specific antibody, and since it is available commercially it presents no difficulties in preparation or standardisation. It would be a further advantage if trypsin were equally effective in the treatment of human serum. However, non-specific and specific inhibitory activities are not so readily distinguished in human as in animal sera, and further investigation will be required before trypsin treatment can be safely applied to them.

SUMMARY.

A study was made of the action of crude *V. cholerae* extracts on the substance (Chu inhibitor) in normal rabbit serum which inhibits haemagglutination by influenza virus. The results suggested that a trypsin-like enzyme known to be present in crude *V. cholerae* extracts was responsible for the inactivation of Chu inhibitor. Crystalline trypsin was found to be highly active in destroying Chu and Francis inhibitors in normal ferret, fowl, rabbit, guinea-pig and mouse sera in conditions where no significant effect on specific antibody was observed.

We should like to thank Drs. C. H. Andrewes and R. Porter for their advice and criticism, and Messrs. E. Owen and D. Eade for their most valuable technical help.

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THE HISTOLOGICAL DEMONSTRATION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN ENDOMETRIUM.

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ENDOMETRIAL alkaline phosphatase activity during normal human menstrual cycles has been described by Atkinson and Engle (1947), Arzac and Blanchet (1948), Hall (1950), and Hughes (1950); with reference to the enzyme localisations in normal tissues, Atkinson and Gusberg (1948) have investigated phosphatase activity of hyperplastic and neoplastic endometria, and Hughes (1950) has investigated cases of sterility and abortion.

The desirability of securing valid localisations of phosphatase activity in normal tissues is emphasised by the contribution of histochemical studies to knowledge of the biological significance of phosphomonoesterases (Moog, 1946). To date histochemical studies of human endometrium have been based on the method of Gomori (1946); recent work (Martin and Jacoby, 1949; Novikoff, 1951) has shown the proneness of this method to artefact, and enzyme localisations described in gland nuclei and in stroma by previous investigators appear unlikely to be valid.

In this investigation phosphatase activity has been studied by (1) a modification of the method of Menten, Junge and Green (1944), in which azo-dye deposition occurs at sites of hydrolysis of sodium α-naphthyl acid phosphate, and (2) the Gomori (1946) glycerophosphate method modified by a × 5 increase of calcium content of the substrate medium and by the incubation of undeparaffinised tissue ribbons. Intracellular enzyme localisations by both procedures are exclusively cytoplasmic, and it is believed are more accurate than those previously reported.

MATERIALS AND METHODS.

Specimens of endometrium were secured from 33 patients aged 18–49 years by curettage or hysterectomy; although uterine abnormalities were present in the majority, all gave a history of regular menses, the cycles in 28 instances being between 24 and 31 days in duration. Blocks fixed in chilled anhydrous acetone were dehydrated in benzene and embedded in paraffin (48°) under vacuum for 1 hour. Paraffin ribbons at 8μ were floated on to substrate solution in covered staining dishes and incubated directly.

Substrate media.

(1) Sodium glycerophosphate (B.D.H.) buffered by sodium veronal at pH 9·3 according to the formula of Gomori (1946). Calcium chloride is present in this solution at a concentration just below 0·01 M.

(2) Sodium glycerophosphate in a similar medium but containing 0·05 M calcium chloride.

(3) Sodium α-naphthyl acid phosphate (Dajac Laboratories) buffered by borax at pH 8·3; the diazonium compound used was Brentamine Fast Blue B (I.C.I. Ltd.). This procedure is identical with the modification by Gomori (1951) of the method of Menten *et al.* (1944).

Incubation in each glycerophosphate medium was for 90 min. at 37°, colour development at sites of calcium phosphate deposition being achieved by the cobalt sulphide method. Incubation in naphthyl phosphate substrate yielded maximal results after 60 min. at 22°. Subsequent rinsing in 1 per cent acid alcohol greatly lessened the brown background caused by decomposition of the diazonium compound, pigment deposits at sites of enzyme action appearing jet black. No counterstaining was employed in either method.

Controls.—Tissues incubated in the presence of 0·01 M KCN showed almost complete enzyme inhibition; the presence of pre-formed calcium deposits was excluded by the von Kossa method. Other tissues mounted wet on albuminised coverslips were deparaffinised and, with or without collodion protection, incubated in conventional manner. Routine hematoxylin-eosin preparations were available for correlation of enzyme localisation with endometrial development.

RESULTS.

(1) Deparaffinised tissues incubated 90 min. in the Gomori medium containing 0·01 M calcium chloride show presumptive sites of enzyme activity similar to those recorded by previous authors; *i.e.*, in early proliferative phase endometrium pigmentation is confined to the free borders of gland and surface epithelium and to capillary endothelium; later it extends through cytoplasm and nuclei of epithelial and to a lesser extent stromal cells. No pigmentation is seen in gland lumina before the 20th day, though after this time it is variably present until the immediate premenstrual phase. Cellular pigmentation during the secretory phase, though greatly reduced, is still apparent.

(2) Deparaffinised tissues incubated in Gomori medium containing 0·05 M calcium chloride show similar pigment localisations to the previous group except that nuclear staining is conspicuously less.

(3) Undeparaffinised tissues incubated in Gomori medium with 0·01 M calcium chloride show even less nuclear staining; in late proliferative and early secretory phase material the stroma, apart from capillary endothelium, is completely unstained. In secretory phase endometrium, pigment deposition is seen in the lumina of many more glands than in corresponding deparaffinised sections.

EXPLANATION OF PLATES.

All photomicrographs of glycerophosphate and azo-dye preparations respectively involved identical normal conditions of exposure and development; the former group were photographed through a green and the latter through a yellow filter, on 35 mm. Ilford Pan F. film. × 300.

FIG. 1.—Proliferative phase endometrium; glycerophosphate; 0·01 M $CaCl_2$; deparaffinised. Dense staining of epithelial nuclei and entire cytoplasm; pigmented contents of lumen represent tangential cutting of gland; stromal staining especially prominent adjacent to gland.

FIG. 2.—Same tissue; glycerophosphate; 0·05 M $CaCl_2$; deparaffinised; staining of epithelial cytoplasm practically restricted to apical border and to nuclei, the latter less intensely stained than those of Fig. 1.

FIG. 3.—Same tissue; glycerophosphate; 0·01 M $CaCl_2$; undeparaffinised; stromal staining absent and nuclear staining slight; unaltered intensity at free border.

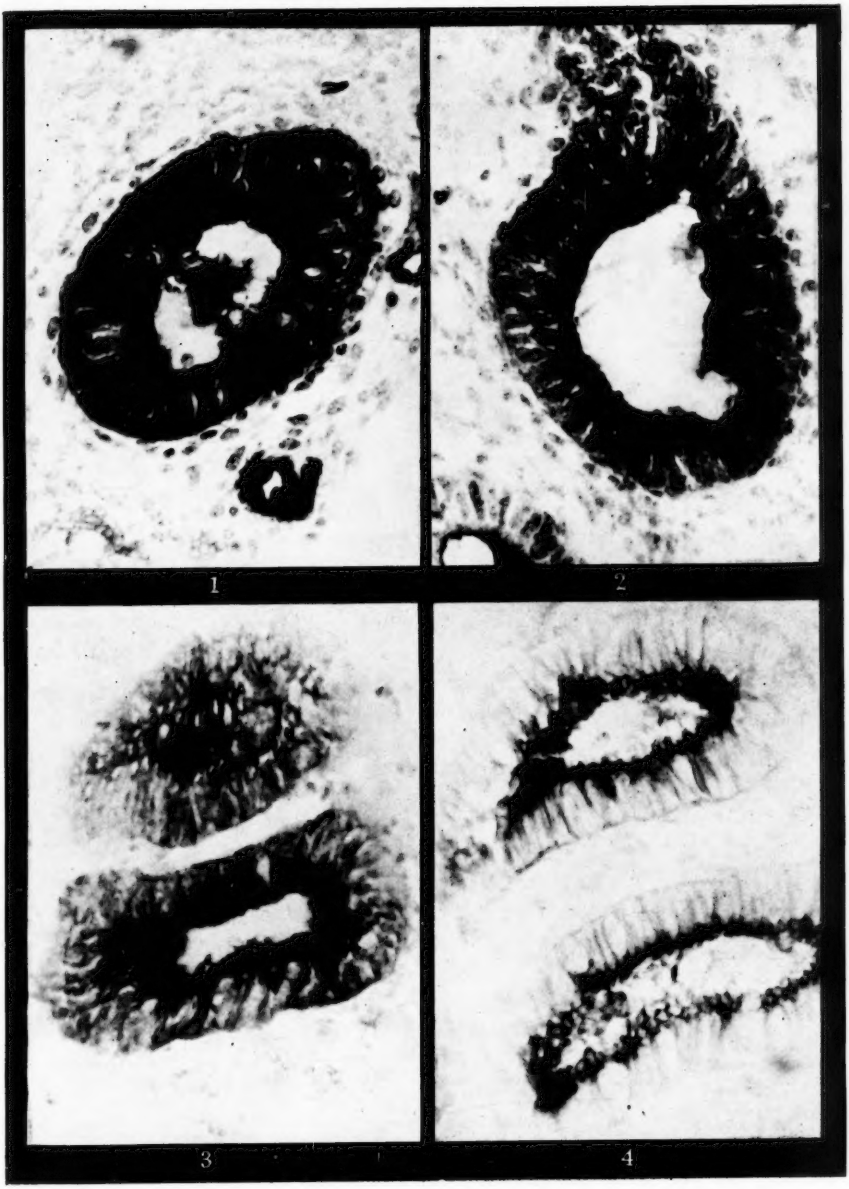
FIG. 4.—Same tissue; glycerophosphate; 0·05 M $CaCl_2$; undeparaffinised; absent epithelial nuclear staining; unaltered intensity at free border.

FIG. 5.—Same tissue; azo-dye; pigmentation restricted to free border of gland epithelium.

FIG. 6.—Early secretory endometrium; azo-dye; pigmentation restricted to free border of gland; secretion not yet apparent.

FIG. 7.—Late secretory endometrium; azo-dye; apparent cellular staining solely the result of yellow decomposition product of diazonium compound; azo-dye pigment localised to gland lumina.

FIG. 8.—Same tissue; azo-dye; staining of endothelium of spiral arterioles.



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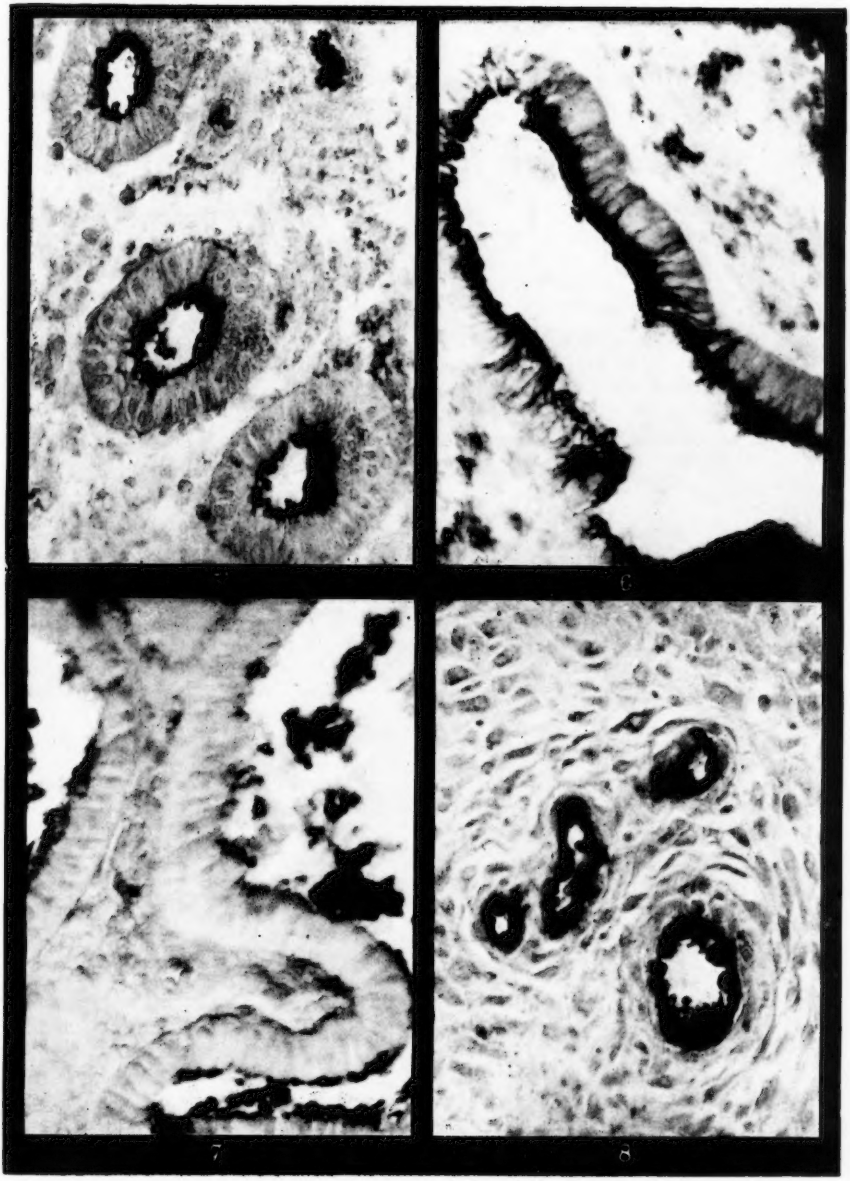

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(4) Undeparaffinised tissues incubated in Gomori medium with 0·05 M calcium chloride show the greatest contrast with the first group; nuclear staining is virtually absent in all phases, and cyclic changes of pigment localisation and intensity are confined to the cytoplasm of epithelial cells, being most intense at the free borders, but occurring to a lesser extent throughout the remaining cytoplasm.

(5) The azo-dye method yields intense black pigment deposits, insoluble in alcohol and xylol, at presumptive sites of enzyme activity. Structures stained include the apical cytoplasm of surface and glandular epithelium, and the endothelium of capillaries and spiral arterioles. Cyclic changes in location and extent of pigmentation resemble most closely those shown by the Gomori method (0·05 M calcium chloride) as applied to undeparaffinised tissues; with the azo-dye method it is immaterial whether tissues are deparaffinised prior to incubation or not.

All methods show cyclic changes of enzyme activity to be most marked in the superficial functional zone of endometrium and there is considerable uniformity from one gland to the next; by comparison glands of the basalis are poorly and irregularly stained in all phases. As previous investigators have found, the extent and localisation of enzyme activity closely parallels in all cases the histological stage of tissue development (Table).

Enzyme Localisations by Modified Gomori and Azo-dye Methods.

Phase.	Surface and gland epithelium.			Stroma.	
	Cytoplasm.				
	Apical.	Basal.	Nucleus.	Lumen.	Vascular endothelium.	Remainder.
Early:						
Proliferative up to 10 days	+	—	—	—	++	—
Late:						
Proliferative 10–16 days	++	+	—*	—	++	—
Early secretory 16–19 days	+++	++	—*	—	++	—
20th day	++++	++	—*	+++	++	—
Secretory 21–26 days	++	—	—	++++	++	—
Premenstrual 27–28th day	—	—	—	+—	+++**	—

* Faint staining only with modified Gomori procedure; completely negative with azo-dye method.

** Endothelium of spiral arterioles prominently stained.

DISCUSSION.

The results of previous investigators have been duplicated in this study by incubation of control deparaffinised tissues for 90 minutes in the Gomori (1946) glycerophosphate medium containing calcium chloride at low concentration (0·01 M); despite the short incubation period intense staining occurs which offers presumptive evidence for cyclical accumulation of phosphatase activity in the nuclei and cytoplasm of glandular epithelium as well as in adjacent stroma. The

experimentally proved capacity of the Gomori method to yield false localisations due to diffusion of calcium phosphate from sites of formation during enzymic hydrolysis of the substrate (Martin and Jacoby, 1949; Novikoff, 1951) makes this demonstration of "nuclear phosphatase" activity appear suspect. Gomori (1951) reported complete absence of diffusion artefact by his method provided, *inter alia*, the incubation period did not exceed 2½ hours and the calcium chloride content of the substrate medium exceeded 0·04 M; deparaffinised sections of endometrium have nevertheless shown appreciable nuclear staining when incubated for only 90 minutes at a calcium level of 0·05 M.

When undeparaffinised ribbons are incubated under the same conditions, pigment deposition is restricted to the cytoplasm of glandular and surface epithelium and to vascular endothelium. Incubation of undeparaffinised tissues, reported by Ruyter and Neumann (1949), was found by Goetsch and Reynolds (1951) to increase the reliability of the Gomori acid phosphatase method; at the completion of the present study a report of Goetsch, Reynolds and Bunting (1952) has appeared indicating absence of nuclear staining when undeparaffinised ribbons of various tissues are examined by the Gomori alkaline phosphatase method. The less intense overall pigmentation in tissues processed by the modified procedure as compared with deparaffinised tissues incubated in Gomori (1946) substrate medium is not explicable by a general reduction of enzymic activity by the altered conditions, for the lowered intensity of staining is apparent only in nuclei and in cytoplasm near the basement membrane; staining of apical cytoplasm and of the contents of gland lumina is completely unaffected.

Intracellular enzyme localisations by the azo-dye method are exclusively cytoplasmic, being maximal at the free border of the cell; consistent failure to stain nuclei might be regarded as evidence of insensitivity, the result of non-optimal conditions of pH (8·3) and temperature (22°) necessitated by instability of the diazonium compound, or of enzyme inhibition due to the latter. However prolonged incubation with repeated changes of substrate does not augment pigment deposits seen after one hour, and Gomori (1951) showed that diazonium salts caused no specific inhibition of nuclear staining when tissues were incubated in his 1946 substrate medium to which these substances had been added.

The foregoing results indicate that the apical cytoplasm of epithelial cells constitutes, for all methods, the main precipitation centre for the insoluble products of intracellular enzymic hydrolysis, staining in more distant structures being the result of diffusion. In the absence of positive evidence to the contrary it may be presumed that maximal intracellular enzyme activity is closely related to the free border, though the theoretical considerations advanced by Johansen and Linderstrom-Lang (1952) suggest that this is not necessarily the case.

The accumulation of phosphatase activity in glandular and surface epithelium during the proliferative phase, its abrupt excretion into gland lumina shortly after ovulation and persistence there until the immediate premenstrual phase appear suggestive of a function other than that of a primary factor in endometrial development; from consideration of its localisations in hyperplastic and neoplastic tissues Atkinson and Gusberg (1948) in fact concluded that the enzyme was not a simple manifestation of endometrial growth. Furthermore, in early human pregnancies endometrial alkaline phosphatase activity has been shown by Wislocki and Dempsey (1945) to disappear completely. The involvement of alkaline phosphatase in carbohydrate and nucleoprotein metabolism together with its

histochemically revealed locations suggests the possibility of a functional rôle in the metabolic processes of the pre-implantation blastocyst.

SUMMARY.

Results of phosphatase staining of sections of endometrium by Gomori's technique and an azo-dye method were compared. It was found that a modified Gomori's technique using undeparaffinised sections eliminated the tendency to artefact in the original procedure.

The enzymic distribution in the endometrial glands during the menstrual cycle showed localisation to the luminal cellular edge in the earlier stages followed by a more uniform distribution throughout the cell at the time of ovulation.

The persistence of phosphatase in the glandular lumen following its discharge after ovulation suggests the possibility of a function additional to that of assisting endometrial development.

I should like to express my gratitude to the British Empire Cancer Campaign Society (Auckland Division), from whom I received a part-time study grant.

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THE EFFECTS OF PHOTO-OXIDATION ON ANTIBODIES.

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Ross (1938), Smetana (1938) and Smetana and Shemin (1941) found that photo-oxidation gradually destroys the ability of antisera to agglutinate or precipitate antigen; they did not consider the possibility that incomplete antibodies might be formed. Ross (1938) found that the abilities of specific antisera to protect against pneumococci and toxin were reduced by photo-oxidation; it appears that the antibodies had not only lost their ability to flocculate, but also their ability to combine with the antigens. However, Tyler and Swingle (1945) found that rabbit antipneumococcal serum photo-oxidized to the point where it became non-precipitating had lost half its protective value, and that horse diphtheria antitoxin photo-oxidized to the point of no longer flocculating with toxin had still the same antitoxic value as the control. It appears that in both cases incomplete antibodies were formed. But in the account of the experiments on which they based this claim Tyler (1945) and Tyler and Swingle (1945) gave no indication of the antigen concentrations, temperature, order of mixing the reagents or of the time allowed for precipitates to form; nor did they give estimates of the amounts of precipitate formed. This work is open to criticism on other grounds which will be discussed later. The possible formation of incomplete antibodies by photo-oxidation has therefore been reinvestigated.

MATERIALS AND METHODS.

Antisera used in these experiments were antipneumococcal (Type I) horse serum and anti-ovalbumin rabbit serum; also a globulin fraction of the horse antiserum prepared by ammonium sulphate precipitation at $\frac{1}{2}$ and $\frac{1}{3}$ saturation. and a γ-globulin fraction of the rabbit antiserum, prepared by Kekwick's (1940) method slightly modified (Marrack, Hoch and Johns, 1951). The Type I pneumococcal polysaccharide (S1) and crystalline ovalbumin were the same as those used in previous work (Orlans, 1952).

The photo-sensitizing dye was water-soluble eosin.

The material to be photo-oxidized was spread in a thin layer in a flat-sided 100 ml. flask, lying on its side, on a shaking platform in a water bath. Small amounts of eosin, up to 0·05 per cent, were added and the flask connected to an oxygen cylinder and a manometer. Illumination was provided by a 100 watt bulb at a distance of 7 cm. from the flask surface. O_2 was passed through the system for 5 min. to remove most of the air and then disconnected. Irradiation and shaking were begun simultaneously. The temperature was adjusted to 20° at the beginning and end of the experiment, when O_2 absorption was measured, and never went above 26°. A slight positive O_2 pressure was maintained throughout irradiation to aid detection of possible leaks.

Three samples of the undiluted horse antipneumococcal serum were irradiated for 90, 140 and 180 min. with an O_2 absorption of 175, 210 and 245 cu.mm./ml. respectively. The horse globulin fraction (3·5 per cent protein) was irradiated 120 min. and absorbed 160 cu.mm./ml., and the rabbit γ-globulin (2·2 per cent protein) absorbed 64 cu.mm./ml. in 85 min. In subsequent discussion the photo-oxidized antisera will be designated by the vol. of O_2 absorbed.

NOTICE TO SUBSCRIBERS.

We have been informed that some of the critical statements in a paper by Eva S. Orlans published in this Journal are erroneous.

We have received the following letter from Mrs. Orlans :—

> "My attention has been drawn to the inaccuracy of my criticism of Dr. Tyler's experimental methods in the introduction to my paper in *Brit. J. exp. Path.*, 1953, **34**, 164. I retract these criticisms and offer my apologies to Dr. Tyler."

As we cannot now withdraw this paper from the record, we request subscribers to be good enough to attach this note to p. 164 of Vol. 34 of the Journal.

The DIRECTOR.

The effect of eosin without irradiation on the precipitation reaction and on the colour development by tyrosine with the Folin-Ciocalteau reagent were investigated and found negligible.

The method of estimating the amounts of ppt. formed by antibody solutions on addition of antigen were those described in an earlier paper (Orlans, 1952).

RESULTS.

Horse antiserum.

As can be seen from Fig. 1, a decrease in the amount of specific ppt. occurred with photo-oxidation and this decrease was proportional to the amount of oxygen absorbed.

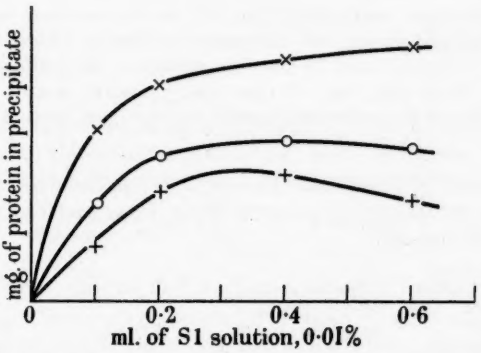

FIG. 1.—Precipitation curves of photo-oxidized and untreated antipneumococcal horse serum.
× ——— × Untreated serum.
○ ——— ○ Photo-oxidized serum (175 cu.mm./ml.).
\+ ——— \+ Photo-oxidized serum (245 cu.mm./ml.).

This decrease in ppt. became much more marked upon storage (in the dark) after exposure to light. For serum 245 the decrease in ppt. was 50 per cent one day after photo-oxidation ; 10 days later it was 90 per cent (Table I*a* and *b*). For

TABLE I.—*Amounts of Precipitate Formed by Untreated and Photo-oxidized Antipneumococcal Serum* (245).

Antiserum.	Volume of antiserum (ml.).	S1 0·1 per cent (ml.).	Precipitate (mg.).	Untreated serum added to supernatant (ml.).	Precipitate (mg.).
(*a*) Photo-oxidized serum ; fresh	0·4	0·1	0·40		
	,,	0·2	0·86		
	,,	0·4	1·04		
	,,	0·6	0·81		
(*b*) Photo-oxidized serum ; after keeping for 10 days	0·4	0·2	0·10	0·4	0·51
	,,	0·4	0·13		
		0·6	0·10	,,	1·23
		0·8	0·05		
(*c*) Untreated serum	0·4	0·2	1·86		
	,,	0·6	1·98		

the horse globulin 160, the precipitin content decreased from 42 per cent of the original to 24 per cent 11 days later.

All irradiated materials were tested for incomplete antibody by one of two methods:

Method A.—Antigen was added to a mixture of photo-oxidized and untreated serum and the amount of ppt. formed was measured. If incomplete antibodies are present in the photo-oxidized serum: (1) they may inhibit ppt. formation with the result that the amount of ppt. formed is less than that formed by the same amounts of antigen and untreated antibody alone, or (2) the amount of ppt. formed may exceed the sum of amounts formed by untreated antibody alone and by photo-oxidized antibody alone.

Method B.—Antigen was added to the photo-oxidized antibody; the ppt. formed was centrifuged down and untreated antibody added to the supernatant fluid. In Table I (lines 5 and 7) there is evidence of incomplete antibody that acts as inhibitor, since the sum of the ppt. (columns 4 and 6) is less than the amount of ppt. formed by untreated serum and antigen (lines 9 and 10, column 4).

TABLE II.—*Amounts of Precipitate Formed by Photo-oxidized Horse Antipneumococcal Serum* (210) (*after keeping* 10 *days*) *Alone and Together with Untreated Antiserum* (*Method A*).

Photo-oxidized serum (210) after keeping (ml.).	Untreated serum (ml.).	S1 0·01 per cent (ml.).	Precipitate (mg.).
0·4	0	0·1	0·69
,,	,,	0·2	0·88
,,	,,	0·4	1·02
,,	,,	0·6	0·93
0·4	0·2	0·1	1·40
,,	,,	0·2	1·68
,,	,,	0·4	2·10
,,	,,	0·6	2·16
0	0·2	0·1	0·93
,,	,,	0·2	0·96
,,	,,	0·4	0·99

On the other hand, in Table IV there is evidence of incomplete antibody that is precipitated with the complete antibody, as the ppt. formed by the supernatant fluid (lines 6, 7, 8, column 6) exceed the ppt. formed by untreated serum and antigen (lines 9, 10, 11, 12, column 4). No incomplete antibody was demonstrated in antisera 175 and 210 (Table II) by method A. The amounts of ppt. formed in the mixture of serum 210 (after keeping) with untreated serum was approximately equal to the sum of the ppt. formed in photo-oxidized serum alone and untreated serum alone.

In serum 245 (after keeping) the presence of incomplete antibodies was shown by method A; in the antigen excess zone the non-precipitating antibody added protein to the ppt. formed with the untreated serum (Table III). Although the addition was considerable it did not account for all the initial antibody, so that it must be assumed that actual destruction of antibody occurred simultaneously with the formation of incomplete antibody. In Table I there is evidence that

TABLE III.—*Precipitates Formed by Mixtures of Photo-oxidized Horse Antipneumococcal Serum (after keeping) with Untreated Serum (Method A).*

Photo-oxidized serum (245) after after keeping (ml.).	Untreated serum (ml.).	S1 0·01 per cent (ml.).	Precipitate (mg.).
0·2	0·2	0·4	0·91
„	„	0·8	1·34
„	„	1·0	1·14
„	„	1·2	0·78
0	0·2	0·1	0·93
„	„	0·3	0·99
„	„	0·4	0·90
„	„	0·8	No precipitate; antigen excess.

TABLE IV.—*Precipitates Formed by* (a) *Untreated Globulin of Horse Antipneumococcal Serum;* (b) *Photo-oxidized Globulin; and Precipitates Formed on Adding Untreated Antiserum to the Supernatant Fluids from* (b); *(Method B).*

Antibody solution.	Volume of antibody solution (ml.).	S1 0·01 per cent (ml.).	Precipitate (mg.).	Untreated antiserum added to supernatant (ml.).	Precipitate (mg.).
(*a*) Untreated globulin	0·4	0·1	1·23		
	„	0·2	1·41		
	„	0·4	1·34		
	„	0·5	1·52		
(*b*) Photo-oxidized globulin (160)	0·4	0·1	0·47	0·2	0·82
	„	0·2	0·51	„	1·14
	„	0·4	0·59	„	1·31
	„	0·6	0·50	„	1·29
Untreated antiserum	0·2	0·1	0·93		
	„	0·2	0·96		
	„	0·4	1·05		
	„	0·6	No precipitate; antigen excess		

incomplete antibody contained in this photo-oxidized serum acted as an inhibitor in the region of antibody excess.

Treatment of serum 245 (after keeping) with ammonium sulphate at $\frac{1}{2}$ and $\frac{1}{3}$ saturation did not result in a separation of the precipitating and incomplete antibodies. Both precipitates were re-dissolved, dialyzed and concentrated to their original vol. by ultrafiltration. The fraction obtained at $\frac{1}{2}$ saturation possessed the same precipitating and inhibitory activity as the whole photo-oxidized serum. The fraction precipitating at $\frac{1}{3}$ saturation was serologically inactive.

Horse globulin 160 possessed no inhibitory activity, but was able to add substantial amounts of protein to a ppt. of antiserum and polysaccharide (method B),

in the equivalence and antigen excess zone (Table IV). Here, too, destruction of antibody and formation of incomplete antibody had taken place at the same time.

In order to detect chemical changes induced by photo-oxidation, parallel Kjeldahl, tyrosine (Folin-Ciocalteau) and quantitative biuret determinations (Mehl, 1945) were performed on the photo-oxidized and untreated sera and globulins, also on the specific ppt. and the supernatants after precipitation with antigen. No changes in the ratio of tyrosine to Kjeldahl N could be detected by these methods. Nor was there evidence of splitting as shown by a decrease in biuret values.

Rabbit γ-globulin.

Immediately after irradiation an *increase* in specific ppt. was observed upon antigen addition (Table V*a* and *b*). Three days later, however, the amount of precipitable antibody had dropped to 23 per cent of that of the original material (Table V*c*), where it appeared to remain constant (re-tested 10 davs and one month later).

TABLE V.—*Precipitates Formed by γ-globulin from Rabbit Anti-ovalbumin Serum* (a) *Untreated;* (b) *and* (c) *Photo-oxidized, Fresh and After Keeping;* (d) *and* (e) *Mixtures.*

	Untreated γ-globulin (ml.).	Photo-oxidized γ-globulin (ml.).	Ovalbumin (mg.).	Precipitate (mg.).
(*a*)	0·4	0	0·037	0·85
	,,	,,	0·074	1·35
	,,	,,	0·148	1·67
	,,	,,	0·222	1·69
(*b*)		Fresh		
	0	0·4	0·037	1·24
	,,	,,	0·074	2·04
	,,	,,	0·148	2·25
	,,	,,	0·222	1·74
(*c*)		After keeping		
	0	0·4	0·046	0·14
	,,	,,	0·074	0·28
	,,	,,	0·091	0·39
	,,	,,	0·148	0·05
(*d*)		Ditto		
	0·4	0·4	0·037	0·07
	,,	,,	0·046	0·46
	,,	,,	0·074	1·65
	,,	,,	0·091	2·38
	,,	,,	0·148	2·99
	,,	,,	0·185	2·90
	,,	,,	0·222	3·10
(*e*)	0·10	0·4	0·091	1·29
	0·15	,,	,,	1·86
	0·20	,,	,,	2·38
	0·30	,,	,,	2·30
	0·40	,,	,,	2·38

Method A was used for (*d*) and (*e*).
All mixtures were kept at 4° for 2 days before estimation of ppt.

Definite inhibition of precipitation was found when antibody was in excess and the mixtures were kept in the cold for two days before estimation of the amount of ppt. (Table V*d* and *e*); if they were left for 5 days the amount of ppt. formed was little less than that formed with untreated globulin alone (Table VI). When the amount of antigen added was not less than the equivalent of the untreated globulin used, the incomplete antibody did not inhibit; on the contrary, the amount of ppt. formed in 2 days with mixtures of untreated and photo-oxidized globulin (Table V*d*) exceeded the sum—amount formed with untreated globulin (Table V*a*) + amount formed with photo-oxidized globulin (Table V*c*). The amount of ppt. (3·10 mg.) obtained from the mixture of 0·4 ml. untreated γ-globulin and 0·4 ml. photo-oxidized γ-globulin with 0·222 mg. ovalbumin is approximately equal to the amount of ppt. that would be obtained from 0·8 ml. untreated γ-globulin with 0·222 mg. ovalbumin. The total antibody protein was therefore not reduced by photo-oxidation. Of this antibody protein the greater part (1·09 mg. in 0·4 ml.) was incomplete.

TABLE VI.—*Effect of Time on Amount of Precipitate Formed by Mixtures of* 0·4 *ml. Photo-oxidized* γ*-globulin from Rabbit Anti-ovalbumin Serum with Varying Amounts of Untreated* γ*-globulin after addition of* 0·046 *mg. Ovalbumin.*

Untreated γ-globulin (ml.).	Precipitate (mg.).	
	After 24 hours.	After 5 days.
0·4	0·25	1·02
0·3	0·14	0·90
0·2	0·16	0·87
0·1	0·05	0·29

Electrophoretic analysis of this γ-globulin before and after irradiation showed no change in the size and shape of the boundary. The mobility, however, was increased to a point half-way between that of γ-globulin and serum albumin. Experiments to demonstrate the combination of non-precipitating photo-oxidized antibody with ovalbumin by electrophoresis were inconclusive, because the mobilities of the antigen and antibody differed so little (owing to the acceleration of the irradiated γ-globulin) and the low mol. wt. of egg albumin.

Chemical analysis of the γ-globulin before and after photo-oxidation, as already described for the horse serum and globulin, revealed no changes.

Spectrographic analysis, however, showed a significant alteration. The ultraviolet absorption of eosin by itself was determined, and found to have no effect on the absorption by protein at the dilutions investigated. The ultraviolet absorption spectra of the untreated and photo-oxidized γ-globulin were determined with a Hilger Uvispek spectrophotometer, at protein concentrations of 0·15 per cent in alkali, in 5 mm. quartz cells (Fig. 2). The tyrosine and tryptophane contents of each were calculated according to Holiday (1936) and Holiday and Ogston (1938) and were:

	Tyrosine.	Tryptophane.
Untreated γ-globulin	124 μg./ml.	55 μg./ml.
Photo-oxidized	129 μg./ml.	33 μg./ml.

These figures indicate that while tyrosine was unaffected, 40 per cent of the tryptophane was destroyed.

FIG. 2.—Ultraviolet absorption curves of untreated (○) and photo-oxidized (+) rabbit γ-globulin. Extinction coefficients, as ordinates, in arbitrary units.

DISCUSSION.

The gradual decrease in precipitating antibody upon photo-oxidation of anti-pneumococcus Type I horse serum and anti-ovalbumin rabbit serum has been shown by Smetana and Shemin (1941). These authors also investigated the chemical changes and the electrophoretic and sedimentation behaviour of the immune sera at various levels of oxygen absorption. Although destruction of histidine and tryptophane was demonstrated during the photo-oxidation of ovalbumin, no such change was found to occur in the antibody protein. The non-coagulable N and amino-N were not significantly affected. The electrophoretic patterns of the whole sera showed a gradual running together of the boundaries, with strong increases in the areas under the boundaries of the faster moving components. The mobilities of a Felton solution of the anti-pneumococcal horse serum and of the rabbit anti-egg-albumin γ-globulin were unchanged, but splitting of the boundaries occurred on prolonged photo-oxidation. Corresponding ultracentrifugal studies of the photo-oxidized rabbit γ-globulin demonstrated the presence of substances of much higher sedimentation rates, which were taken to be aggregation products of γ-globulin. The possibility of incomplete antibody formation was not considered by Smetana and Shemin. They did not observe an increase in the amount of precipitate formed with antigen added to antibody solution, immediately after photo-oxidation, such as was found with rabbit γ-globulin (Table V); nor did they observe the progressive reduction of the amount

of precipitate that occurred when the treated antibodies were kept in the dark after photo-oxidation (Tables I and V). This progressive reduction must have been due to slow changes that took place after activation by irradiation. This may be compared with the progressive loss of activity of pepsin after exposure to X-rays (Anderson, 1947, 1952).

The investigations of the photo-oxidation of antisera by Tyler (1945) and Tyler and Swingle (1945) were undertaken with the specific aim of producing "univalent" antibodies, which they claimed to have achieved. This work is open to criticism, not only on grounds of theoretical interpretation, but also on those of experimental results. The degree of oxidation, as measured by O_2 absorption, required to destroy the visible reactions of antisera as reported by Tyler was a great deal lower than the values found by Smetana and Shemin and ourselves. Tyler found that isolated globulin fractions were resistant to photo-oxidation and coagulated on prolonged irradiation; electrophoretically homogeneous rabbit γ-globulin as well as horse globulin fractions were photo-oxidized without difficulty by other workers. The claim of separating "multivalent" and "univalent" antibodies from non-precipitating sera by fractional salting out with ammonium sulphate at $\frac{1}{2}$ and $\frac{1}{3}$ saturation respectively was not confirmed in our experiments.

Tyler's contention that his non-precipitating antibodies are univalent is supported by none of his experiments. The very magnitude of the inhibitive titres (in the case of an antipneumococcal Type III serum with an O_2 uptake of 307 c.mm./ml., 0·4 ml. photo-oxidized serum sufficed to prevent the precipitation of 1·0 ml. untreated serum) speaks against univalence.

Boyd (1946), working with photo-oxidized anti-A and anti-Rh isohaemagglutinating antibodies, was not able to demonstrate the presence of any inhibitory antibody. He found that irradiation merely produced gradual inactivation. The conditions of his experiments, however, were somewhat different, since he did not irradiate in an oxygen atmosphere and with shaking, and used photo-floods which produce a more intense light with shorter wave-lengths. Since the actions of ultraviolet and visible light in the absence or presence of O_2 are known to induce different reactions in proteins the discrepancy is not surprising.

From our experiments we conclude that, under certain conditions, at least, incomplete antibody formation occurs together with actual destruction of antibody proteins in the course of photo-oxidation. Whether the two processes are simultaneous or successive cannot be said.

Although the non-precipitating antibody obtained by this method can delay considerably the flocculation of untreated antibody with antigen, it is not an inhibitor in the sense of a hapten. It is, however, incomplete in that, although unable to precipitate by itself, it can co-precipitate in definite proportions with homologous precipitating antibody and antigen.

These experiments show that the effects of incomplete antibody on the amount of precipitate formed vary with conditions. In the first place the formation of precipitate may be much delayed, so that much more may be found after 5 days than after one day. In the second place, the incomplete antibody may inhibit in the region of antibody excess and co-precipitate in the region of antigen excess. As was already found (Orlans, 1952) with lipid-extracted sera a considerable amount of the incomplete antibody is precipitated by antigen from a mixture of a small amount of untreated antibody with the photo-oxidized antibody.

The mode of formation and nature of these antibodies can only be guessed at this stage. At least two and probably more distinct reaction mechanisms are involved in their formation, namely, the chemical changes induced in individual amino-acids by O_2 absorption, and the physical interaction of the protein components in the serum. The absorption of O_2 upon irradiation with visible light in the presence of a photo-sensitizer has been shown to be a general reaction of conjugated double bonds in a ring system (Bergmann and McLean, 1941) analogous to the addition of maleïc anhydride. It results in the opening of the ring and the formation of two carbonyl- or hydroxyl-groups, depending on the oxidation-reduction properties of the medium. Five-membered rings are far more reactive in this process than six-membered ones, and one would expect that the imidazole and indole groups would be the most likely to be affected. This is confirmed by the results of the chemical analysis of Smetana and Shemin (1941), who found that in egg albumin only histidine and tryptophane were destroyed by photo-oxidation. Since histidine absorbs very weakly in the ultraviolet we were not able to test for its disappearance, but the destruction of tryptophane and the conservation of tyrosine were confirmed. Increased electrophoretic mobility and greater solubility in ammonium sulphate would result from an increase in hydroxyl-groups.

As the nature of the protein interactions depends on the proteins present, different results are to be expected from the photo-oxidation of whole sera and of isolated globulin fractions. In the case of whole sera, aggregation of γ-globulin with albumin has been amply demonstrated. This aggregation is similar to that occurring in heat-treated antisera, and should suffice to account, through increased solubility, for the properties of these incomplete antibodies.

When γ-globulin is photo-oxidized alone an increase in solubility must be due to the formation of more polar groups. There are strong indications, however, that this is not the only process involved. The ultracentrifuge results of Smetana and Shemin and the initial increase of the precipitable protein (Table V) point to the formation of aggregates. But, if such aggregation of antibody protein with other protein occurs, the equality of the total antibody protein in the photo-oxidized γ-globulin to the antibody protein of the untreated γ-globulin might be an accidental coincidence due to simultaneous aggregation and destruction of part of the antibody. It is possible that these " complexed " antibodies are univalent either for steric reasons, or through the loss of a combining group due to tryptophane or histidine destruction. The evidence does not suffice to discard solubility changes in favour of univalence to explain the behaviour of photo-oxidized incomplete antibodies.

The effects of irradiation with ultraviolet light, described by Hanan (1952) in a paper which appeared after this work was finished, differ from the effects of photo-oxidation. The chief difference was that after ultraviolet irradiation the maximum precipitate was obtained with low ratios of antigen to antiserum and that there was little evidence of aggregation.

SUMMARY.

A progressive change takes place in photo-oxidized antibodies when they are kept in the dark after irradiation.

Immediately after photo-oxidation of horse antipneumococcal serum the

amount of precipitate formed with antigen was not reduced; after keeping in the dark incomplete antibodies were formed but there was little evidence of actual destruction of antibody.

Immediately after irradiation of the γ-globulin of rabbit anti-ovalbumin serum the amount of precipitate formed with antigen was increased; after keeping incomplete antibodies were formed and some antibody was destroyed.

Precipitates are formed slowly after addition of antigen to mixtures of untreated antiserum with photo-oxidized antibody; precipitation may appear to be inhibited unless the mixtures are kept for several days.

The effects of incomplete antibodies on the amount of precipitate formed with antigen and untreated serum vary with conditions; the amount of precipitate may be reduced in the region of antibody excess and increased in the region of antigen excess.

The change in the ultraviolet absorption curve suggests that the tryptophane of serum proteins was affected by photo-oxidation and that the tyrosine was unaffected. It is suggested that photo-oxidation mainly affects the 5-membered rings of tryptophane and histidine. There is evidence that the γ-globulin of rabbit serum is aggregated after photo-oxidation.

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THE INHIBITORY EFFECTS OF CHOLESTEROL AND RELATED STEROLS ON HAEMOLYSIS BY STREPTOLYSIN O.

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THE well-established observation that aqueous emulsions of cholesterol can protect mammalian red cells from lysis by certain bacterial and steroid haemolysins (van Heyningen, 1950) has suggested that the action of these toxic substances may depend on their forming some disruptive combination with the cholesterol of the cell envelope. For some bile acids, and especially for lithocholic acid, the mode of inhibition of haemolytic action by sterol compounds has been clarified by the work of Berliner and Schoenheimer (1938), who found that among those they examined, protective power was characteristic of sterols possessing a hydroxyl radicle at position 3 *cis* (or β-) to the methyl group at position 10 and accompanied by either a *trans* fusion of rings A and B or a double bond between the 5 and 6 carbon atoms. It appeared from this study that the neutralization of this type of haemolysin by its sterol inhibitors involved interactions of a closely-defined stereochemical nature. The present investigation has been made to determine, firstly, whether the action of the bacterial toxin streptolysin O can also be inhibited by sterols other than cholesterol, and secondly, if so, whether this property is associated with any comparably specific configuration of the molecules of these substances.

Before describing this work, it is necessary to point out that we have departed significantly from the techniques used by earlier workers in that the potentially inhibitory sterols are not presented to the streptolysin in a finely suspended particulate form but in an orientated manner as a film at a ligroin-water interface. There are two reasons for this modification: to lessen the likelihood that any recorded inhibition might result from some non-specific behaviour of the lysin on the surface of the particles in the emulsion, and to bring the hydrophilic radicles of the sterol molecules—notably the hydroxyl group at position 3—most intimately into relation with the solutes present in the aqueous phase. It was hoped that were neutralization of the streptolysin under these conditions found to be associated with some particular configuration of the hydrophilic radicles of the sterols examined, it would advance our knowledge of the mode of action of this important toxin.

MATERIALS AND METHODS.

Definition of a "Haemolytic Unit."—A solution of streptolysin O was regarded as containing one haemolytic unit (H.U.)/ml. when it produced 50 per cent haemolysis in an equal volume of a 2·5 per cent suspension of rabbit's red cells in 30 min. at 37°. Thus, for example, if this degree of haemolysis was produced by a 1 : 100 dilution of the lysin, the preparation would be regarded as containing 100 H.U./ml.

Preparation of streptolysin O.—This toxin was produced by growing the "Lambert" strain of *Streptococcus pyogenes* (Group A) in the semi-synthetic medium described by Bernheimer, Gillman, Hottle and Pappenheimer (1942). After sterilization according to their technique, 15 l. of the medium were distributed in four 5 l. conical flasks and inoculated with the saline-resuspended deposit from a 6 hr. culture of the organism grown at 37° in glucose broth. The flasks were incubated at 37°, and after the first 15 hr. of growth the increasing acidity of the cultures was neutralized by the frequent addition with gentle agitation of small amounts of 7·5 M NaOH.

After 28–30 hr. incubation, 1/10th of their volume of anaesthetic ether was added to the flasks to kill the organisms, and the cultures kept at 2° overnight. The following day the bacteria were removed in a Laval centrifugal separator. At this stage the streptolysin titres of the cultures varied slightly, the average being 20 H.U./ml. For further concentration the clarified supernatant was suspended in cellophane sacs (Visking transparent cellulose tubing), against which an air current was directed. After reduction in bulk from 1/5th to 1/8th, the preparation was dialysed overnight against running tap-water. After concentrating thus, the titres of the various batches varied from 75 to 160 H.U./ml. Merthiolate 1 : 10,000 was added as a preservative, and the sterility of the preparations checked at intervals. Through the kindness of Dr. Humphrey and Mr. Ward, of the National Institute for Medical Research, some of the preparations were freeze-dried and stored *in vacuo*. It was found, later, however, that concentrated solutions of the streptolysin were stable for at least 4 months when kept at 2°.

Estimation of haemolytic titres.—(a) *Reducing agent:* Sodium thioglycollate was used for the re-activation of the streptolysin O after its reversible oxidation, 0·1 ml. of a 90 per cent solution of thioglycollic acid being diluted with 2 ml. distilled water, a few drops of B.D.H. Universal Indicator added, and 7·5 M NaOH added drop by drop until the solution turned blue-green (pH 6–7). When made up to 5 ml. with saline-phosphate buffer (see below), this gave an approximately 0·2 M solution. (b) *Saline-phosphate buffer:* All haemolysis estimations were made in the isotonic saline-phosphate buffer described by Herbert (1941). (c) *Red cell suspensions:* Fresh rabbit's blood was collected in 3 per cent sodium citrate: the deposited cells were washed three times in the saline-phosphate buffer before being made up into a 2·5 per cent suspension in the same solution. The suspension was always freshly prepared on the day of use. (d) *Haemolysis:* Estimations were made in 15 ml. conical centrifuge tubes. Five ml. of the reducing solution was added to 20 ml. of the streptolysin solution, and the mixture allowed to stand at room temperature for 10–15 min. before use. For titration, falling dilutions of the lysin were prepared. To 1·5 ml. of each dilution, 1·5 ml. of the buffer and 3 ml. of the red cell suspension were added, and the tubes immersed in a water bath at 37° for 30 min. The tubes were then centrifuged, and the haemoglobin concentrations of the supernatants determined in a "Spekker" photo-electric absorptiometer, for which a calibration curve for haemoglobin solutions had previously been prepared. To exclude the possible presence of any oxygen-stable haemolysin (streptolysin S), initial titrations of the haemolysin solutions were carried out after the addition of potassium persulphate (about 0·5 ml. of an 0·1 M solution).

Presentation of the sterols to streptolysin O.—The sterols were obtained through the kindness of various donors and were used in the pure state. Berliner and Schoenheimer (1938) found that the unsaturated sterols, especially cholesterol, readily undergo oxidation at the double bond, giving highly heaemolytic substances which on re-crystallisation revert to their former protective form. In the present experiments all the cholesterol used was re-crystallised from absolute alcohol on the day of use. The solvent used to carry the sterols and steroids was ligroin (B.D.H., b.p. 90–120°).

The solution of the sterol or steroid in ligroin (0·1 per cent) was presented to the streptolysin solution in its aqueous buffer in the apparatus depicted in Fig. 1. This apparatus consisted of a U-tube with a reservoir at the top of both limbs. A tap about midway down the right-hand limb controlled the rate of flow of the ligroin solution. The wider left-hand limb had a fine nozzle 5 cm. from its lower end, through which small drops of the ligroin solution issued and began their slow ascent through the column of the streptolysin solution. Before use the apparatus was washed with ligroin. The sterol-ligroin solution was then introduced into the right-hand limb and allowed to flow through the apparatus until it reached the nozzle. The tap was then closed, and the lysin-containing limb washed out, first with saline-buffer and then with 1 ml. of the activated streptolysin solution. Five ml. of the streptolysin solution was run into this limb, and the control tap then opened until

the ligroin-sterol solution entered through the nozzle at the rate of 1 to 4 drops/sec. The ligroin-sterol solution collecting above the lysin column was removed at intervals, and fresh solution added to the reservoir at the top of the right-hand limb to maintain a continuous flow. After 50 ml. of the ligroin-sterol solution had been allowed to pass in this way, the streptolysin solution was removed for titration of its residual haemolytic activity. In every experiment, the haemolytic activity of a control solution of activated streptolysin which had stood for the same time at room temperature was also estimated, and the degree of inhibition of the perfused lysin expressed in relation to this control.

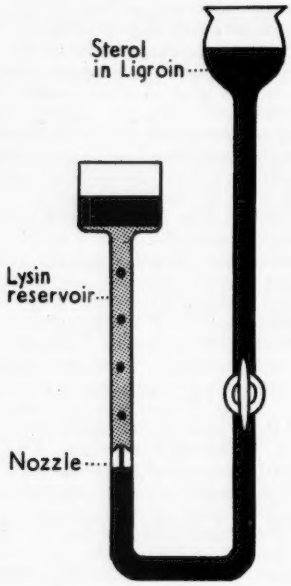


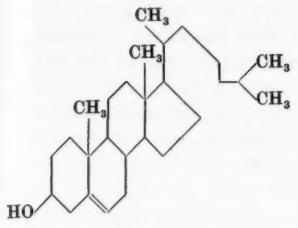
FIG. 1.—Diagram showing apparatus for passing ligroin-sterol solutions through the streptolysin reservoir.

Since it was possible that some loss of the streptolysin might occur in consequence of adsorption of the protein to the glass wall of the apparatus, the activity of a column of the lysin was determined after it had stood in the left-hand limb at room temperature for 1 hr.; no decrease in haemolytic activity was found. The specific character of the neutralization was further tested by passing ligroin alone through a column of the streptolysin; no fall in titre was found to follow.

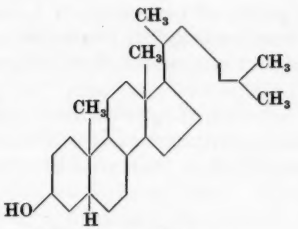
Benzene and chloroform were also considered as solvents for the sterols: the former was unsatisfactory in failing to dissolve all the compounds examined, while the higher specific gravity of the latter precluded its use in this type of apparatus.

RESULTS.

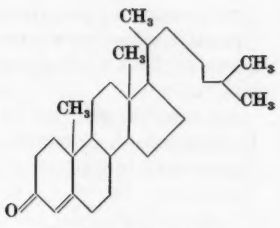
A list of the sterols and steroids examined together with their chemical configuration and their neutralizing capacities for streptolysin O are given in Fig. 2. With the exception of dehydroisoandrosterone, all the sterols with a β-hydroxyl group at carbon atom 3 were potent inhibitors of the lysin. Cholesterol, stigmasterol and cholestanol were all strongly inhibitory, ergosterol and



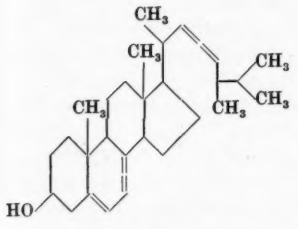
I. Cholesterol (β). +++.



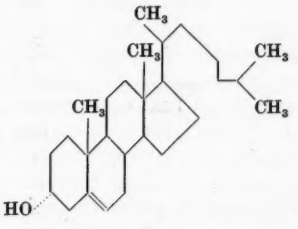
V. Coprostanol (β). ++ (Coprosterol).



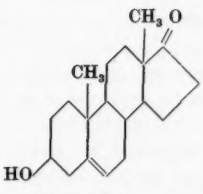
VIII. Cholest-4-en-3-one (keto). −.



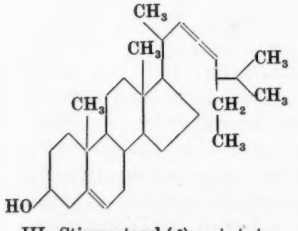
II. Ergosterol (β). ++.



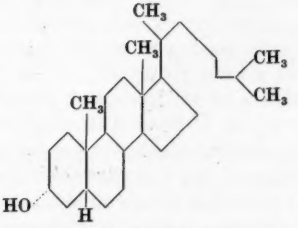
VI. Epicholesterol (α). −.



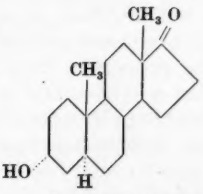
IX. Dehydroisoandrosterone (β). −.



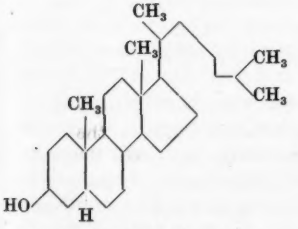
III. Stigmasterol (β). +++.



VII. Epicoprostanol (α). −. (Epicoprosterol).



X. Androsterone (α). −.



IV. Cholestanol (β). +++ (Dihydrocholesterol).

FIG. 2.—Names and structural formulas of the various sterols and steroids, together with the arrangement of the hydroxyl group at carbon atom 3 (α or β) and degree of inhibition of streptolysin O (−, no inhibition; ++, 50–75 per cent; and +++, > 75 per cent inhibition).

coprostanol rather less so. In contradistinction to these, none of the sterols that possess an α-hydroxyl group at carbon atom 3 effected any inhibition. The steroid cholest-4-en-3-one was included in the series to test the effect of a keto instead of an hydroxy group at carbon atom 3: this substance caused no inhibition.

A lecithin solution (0·1 per cent of egg lecithin in ligroin) also failed to inhibit. It was found, however, that a mixture of lecithin and cholesterol dissolved in ligroin was inhibitory, though less so than cholesterol alone.

DISCUSSION.

The ability of cholesterol and other kindred sterols to inhibit the haemolytic activity of a solution of streptolysin O when brought into relation with it across an oil-water interface appears to depend upon certain distinctive features in the steric configuration of their molecules. The inhibitory sterols are cholesterol, stigmasterol, cholestanol, ergosterol and coprostanol, all of which resemble one another in possessing a β-hydroxyl group at carbon atom 3 (C 3) of the cyclopentanephenanthrene nucleus (*i.e.*, a hydrophilic polar group) and a hydrophobic group attached to carbon atom 17 (C 17) at the other extremity of the molecule. The other sterols and steroids studied, all of which failed to neutralize the streptolysin, differed from this steric arrangement in various respects. Epicholesterol and epicoprostanol both possess the same hydrophilic hydroxyl and hydrophobic groups attached at the same sites in the molecule, but both of them have the α-arrangement, a steric configuration which seems to preclude its presentation across the interface to couple with the reactive groups of the lysin molecule. Cholest-4-en-3-one possesses a keto group at C 3—a polar group that has widely differing properties from those of an hydroxyl group. Dehydroisoandrosterone possesses a β-hydroxyl group at C 3, and in this respect resembles cholesterol and the other inhibitory sterols, but the hydrophobic group at C 17 that is typical of these compounds is replaced by a hydrophilic polar group whose presence would greatly modify the orientation of the molecule at such an interface. Androsterone fulfils neither of the above conditions that seem to be required for complex formation with the streptolysin, since the hydrophilic hydroxyl group at C 3 has the α-arrangement and the molecule possesses no hydrophobic group at C 17.

That the ability of certain sterols to inhibit the haemolytic action of streptolysin O depends upon the steric arrangement is supported by some observations made with other haemolysins. Cohen, Shwachman and Perkins (1937) found that the activity of the related oxygen-labile pneumococcal haemolysin is inhibited by much smaller concentrations of the digitonin-precipitable sterols, cholesterol, cholestanol and, to a lesser degree, coprostanol than are required for the digitonin-non-precipitable sterols. Ponder (1945), in a study of haemolysis produced by digitonin and saponin, found that the inhibition exerted by emulsions of cholesterol and its related sterols depended primarily upon a normal (or β-) as opposed to an epi- (or α) configuration of the hydroxyl group at C 3. It would seem, therefore, from these studies, as well as from that of Berliner and Schoenheimer already mentioned, that all these bacterial and glucosidal haemolysins are capable of neutralization by sterols that possess the same distinctive steric configuration at this site in the molecule. From this common characteristic,

it would seem likely that they possess some common mode of attack on the red cell envelope.

Although details of the structure of the red cell envelope are still obscure, it is now known to be composed of closely associated molecules of cholesterol, phosphatides and protein (Frey-Wyssling, 1948; Ponder, 1945). According to Winkler and Bungenberg De Jong (1940–41) and Booij (1949), the most superficial layer is a palisade-like film of orientated phosphatides and cholesterol, the molecules of the latter lying between and stabilizing the more highly dissociated and mutually repulsive phosphatide molecules. Were this so, the cholesterol in the envelope would be freely accessible to lysins in the surrounding medium, and the disruption of the envelope could be accomplished by penetration of its surface film. This conception of the mechanism of haemolysis by lysins of the type here considered is supported by observations of Schulman and Rideal (1937) on interactions between saponin and stable cholesterol-protein monolayers. They found that the factors which determine the penetration of such films are the same as those that determine complex-formation in these mixed monolayers: first, the polar heads of the two types of molecule must mutually interact, and second, there must be a hydrophobic tail to the film-forming molecule which must adhere or interact by van der Waal's forces with some hydrophobic group in the penetrating molecule. When only the polar interaction between the two substances occurs, no penetration of the film follows. It can be seen that this concept of interaction defines criteria that accord with our findings upon the characteristics required in the sterols studied for inhibition of streptolysin and by others for the inhibition of kinetic-lytic agents, *viz.*, a specific hydrophilic hydroxyl group configuration at one end of the sterol so arranged as to be able to couple with the lysin and a hydrophobic group at the other.

SUMMARY.

The inhibition of the haemolytic activity of streptolysin O by cholesterol and closely related sterols has been studied by presenting the sterol to the lysin at a ligroin-water interface.

Inhibition of activity was effected by sterols which possessed a hydrophilic hydroxyl group at position 3 of the sterol nucleus in *cis* (or β-) position to the methyl group at position 10 and a hydrophobic group attached at position 17 (cholesterol, ergosterol, stigmasterol, cholestanol and coprostanol). Other sterols in which these criteria were not fulfilled caused no inhibition of haemolytic activity.

It is suggested that the inhibition of haemolysis is due to complex formation between the lysin and the sterol molecules when the latter are orientated at the interface, and that the lysis of red cells by streptolysin O is dependent on complex formation between the lysin and the cholesterol of their envelope with resultant penetration of the surface film.

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THE COMPARATIVE RESISTANCES OF THE RED CELLS OF VARIOUS SPECIES TO HAEMOLYSIS BY STREPTOLYSIN O AND BY SAPONIN.

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ALTHOUGH the comparative resistances of the red cells of various species to lysis by saponin and other haemolytic agents have been investigated (Rywosch, 1907 ; Ponder, Saslow and Yeager, 1930), no comparable observations for streptolysin O have yet been made. In view of the probable importance of this lysin in the lethal intoxications resulting from inoculation with streptococcal filtrates, the present study was made to find out how far the red cells of some common laboratory animals differ in resistance to its lytic action. Since the kinetics of haemolysis for nucleated and non-nucleated red cells are essentially the same (Shattuck, 1928), the range of species examined was extended to include red cells of the former as well as the latter types. Further, because of certain similarities between the modes of action of streptolysin O and saponin, notably their common neutralizability by cholesterol, comparative resistances were determined for both lytic agents.

MATERIALS AND METHODS.

Streptolysin O.—This lysin and its thioglycollate reducing solution were made in the manner described in the preceding paper (Howard, Wallace and Payling Wright, 1953).

Red cell suspensions.—The rabbit was used throughout as the reference species against which the others were compared. Fresh blood, collected in 3 per cent sodium citrate solution, was washed thrice in Herbert's buffer-saline solution and finally made up to a 2·5 per cent suspension in the same fluid. For studying the action of lysins on red cells of such differing sizes, suspensions based on constant percentages of packed cell volumes are preferable to those based on fixed cell counts.

Estimations of haemolysis.—(a) *Streptolysin :* Serial dilutions of this lysin were made in buffer-saline solution : to 1·5 ml. of each were added a further 1 ml. of buffer saline and 0·5 ml. of sodium thioglycollate solution. The mixture was placed in a water bath at 37° for 10 min. to reduce the lysin ; 3 ml. of the appropriate red cell suspension was then added to each tube. After a further 30 min. incubation the tubes were centrifuged and the percentage haemolysis determined with a " Spekker " photoelectric absorptiometer. (b) *Saponin :* Serial doubling dilutions of saponin in normal saline were made from an initial solution containing 200 μg./ml. ; fresh preparations were made for each haemolytic series since the activity of saponin deteriorates in solution. To 3 ml. of each dilution of saponin was added 3 ml. of the appropriate red cell suspension, and after the tubes had been immersed for 30 min. in a water bath at 37° the percentage haemolysis was determined as for the streptolysin.

Rabbit's cells were used as the reference for those of other species, which were examined two or three at a time. Resistances to streptolysin and to saponin were tested on the same day to improve comparability of results. The same two rabbits were used throughout the experiments.

Comparison of results.—The dilutions of streptolysin and of saponin that produced 50 per cent haemolysis of rabbit's cells under these conditions were regarded as containing one

haemolytic unit (H.U.)/ml. From this defined standard the number of units needed to produce the same degree of haemolysis with the other species was derived. With the preparation of saponin used, 1 H.U. was equivalent to 15 μg./ml.

RESULTS.

From the comparative resistances to haemolysis shown in Table I, it can be seen that streptolysin O is active against the cells of all the species examined.

TABLE I.—*The Comparative Resistances of the Red Cells of Various Species to Lysis by Streptolysin O and by Saponin.*

Species.	Mean red cell diameter (μ).*	Number of H.U. for 50 per cent lysis.	
		Streptolysin O.	Saponin.
Rabbit	7·0	1·0	1·0
Human	7·4	0·25	1·25
Guinea-pig	7·2	1·0	1·5
Dog	7·1	0·75	4·0
Rat	6·8	2·0	0·75
Mouse	6·7	> 32	0·75
Hamster	6·6	1·5	1·0
Ox	6·0	0·75	4·0
Pig	6·0	0·75	1·0
Cat	5·8	1·0	2·0
Horse	5·5	0·75	2·0
Sheep	4·8	0·75	4·0
Goat	4·0	1·5	4·0
Duck	13·1×7·4	1·0	0·75
Pigeon	12·9×7·0	1·5	1·0
Chicken	12·1×7·3	5·0	0·75
Frog	23·0×14·0	2·0	1·0

* Data from Gulliver (1846) and Wintrobe (1951).

Those of mice, however, possess a resistance of a wholly different order from the relatively narrow range found with the other 16 species. At a concentration of 32 H.U./ml. only 10 per cent haemolysis had occurred; 50 per cent haemolysis was never attained with this species because sufficiently potent preparations of streptolysin were not available. That this high resistance was not peculiar to any particular group of mice (to obtain enough cells for each test, blood from several mice was pooled) is shown by finding consistent results from batches of animals drawn from each of two laboratory strains. Of the other species, the cells of the hamster, rat and goat were rather more, and those of the horse, ox, sheep, dog, pig and human rather less resistant than those of the rabbit. Cat and guinea-pig cells had about the same resistance as rabbit cells. Of the nucleated cells examined, those of the fowl were much more, and those of the pigeon rather more, resistant than rabbit's cells.

The red cells of none of the species presented the same striking resistance to lysis by saponin as had been found for mouse cells by streptolysin. Several species required higher concentrations of saponin to effect an equivalent degree of lysis, but with most, the resistance did not differ much from that found for the rabbit.

It is of interest that when the species are listed in two series according to resistances to the two lysins, some degree of negative correlation emerges.

DISCUSSION.

We have no explanation for the exceptional resistance of mouse red cells towards streptolysin O. Although their resistance to this toxin has not been described before, there are records of their high resistance to two other oxygen-labile, cholesterol-inhibitable, haemolysins, *viz.*, pneumolysin and *Cl. welchii* θ-toxin. Cohen, Halbert and Perkins (1942) found that mouse red cells withstood ten times the concentration of pneumolysin that lysed those of the rabbit, sheep or man, and Oakley (1943) noted that *Cl. welchii* θ-toxin "is vigorously haemolytic for the red cells of most laboratory animals except those of the mouse, which are only slightly attacked." Since it seemed possible that the relative resistance of mouse cells might extend to all four known haemolysins of this type, we compared the lysabilities of mouse and rabbit cells to two preparations of tetanolysin. With this toxin the resistance of mouse cells was less distinctive; they were only four times more so than rabbit cells. It would nevertheless seem that mouse erythrocytes possess some constitutional feature rendering them less vulnerable than those of other common laboratory species to lysins of the oxygen-labile, cholesterol-inhibitable, kind.

The exceptional resistance of mouse cells does not seem to depend on any distinctively different cholesterol content, for we have determined the concentrations of this sterol in packed mouse, guinea-pig and rabbit red cells and found them to be 190, 180 and 155 mg./100 ml. respectively. Moreover, were a high cholesterol content of the cell envelope responsible for the differences in resistance to streptolysin O, mouse cells might be expected to be similarly resistant to saponin, which they are not. Without discarding the possibility, however, that lysis by streptolysin O ultimately depends on a complex formation with cholesterol in the envelope, two possible explanations might account for this lack of correlation. The cholesterol in the cells of some species may be more accessible to this lysin than is that in mouse cells, or in these latter cells a higher proportion of the cholesterol may be in esterified form.

We have applied three tests in the hope of seeing whether mouse red cells are distinctively protected against the streptolysin molecules by some surrounding protein film. The lysin was allowed to act on cells previously treated with trypsin (Morton and Pickles, 1951); no change in resistance followed. A second test was based on the observation of Mudd and Mudd (1926) that after the attachment of a film of agglutinating protein rabbit red cells lose their ability to pass through an oil-water interface; the comparative behaviours of normal mouse and rabbit cells at such a boundary was examined, and both appeared to have the same hydrophobic properties. Thirdly, the possibility that the source of the high resistance of the mouse cells might be found in the participation of some protein constituent of their serum seems to be lessened by the finding that erythrocytes of mouse and rabbit after transference to, and incubation for one hour in, the serum of the other species, undergo no sensible change in their relative resistances to streptolysin O.

Finally, before any further attempt is made to analyse this species idiosyncracy, it may be pointed out that the enhanced resistance of mouse erythrocytes to streptolysin O seems not to be peculiar to this type of cell, for, as will be shown in the following paper, mice are also much more resistant to the lethal action of this toxin than either rabbits or guinea-pigs.

SUMMARY.

The comparative resistances of the red cells of 17 common laboratory animals to haemolysis by streptolysin O and saponin have been determined. Although the cells of all these species can be lysed by both agents, mouse cells were found to be exceptionally resistant to streptolysin O.

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THE COMPARATIVE RESISTANCES OF RABBITS, GUINEA-PIGS AND MICE TO THE LETHAL ACTIONS OF STREPTOLYSIN O AND SAPONIN.

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SINCE the differentiation of streptolysins O and S by Todd (1938*a*), it has been recognized that preparations containing the former are rapidly lethal for mice when injected intravenously (Todd, 1938*b*; Hewitt and Todd, 1939). It is still uncertain, however, whether the haemolytic and lethal activities of such preparations are due to the operation of the same or different agents. Harris (1942) took the latter view, for he found that the destruction of the haemolytic activity of his preparation of streptolysin O by heat, tryptic digestion, neutralization by anti-streptolysin, dialysis and exposure to cholesterol emulsions did not diminish its lethal power. We have re-investigated this question, making use of most of these differentiating tests for lytic and lethal activities; our results are not compatible with Harris's conclusions.

Because mouse red cells are exceptionally resistant to streptolysin O *in vitro* (Howard and Wallace, 1953), the lethal power of this toxin has been studied in three species, mouse, guinea-pig and rabbit. Further, since saponin also possesses both haemolytic and lethal powers for these three species, a parallel study has been made on the lethal action of this glucoside. Finally, in view of the strong affinity of streptolysin O for cholesterol, it was decided to produce hypercholesterolaemia in a group of rabbits by injections of " Triton WR 1339 " to determine whether a raised concentration of this sterol in the blood was followed by increased resistance to the lethal action of streptolysin.

MATERIALS AND METHODS.

Preparation, activation and titration of streptolysin and titration of saponin.—These procedures were carried out as previously described (Howard and Wallace, 1953) with the following modifications: The streptolysin was not dialysed after concentration except when the effect of dialysis on the lethal factor was studied. For all *in vivo* experiments, 1 ml. neutralized sodium thioglycollate was added to 9 ml. streptolysin solution and allowed to stand for 15 min. at room temperature. The lysin was not diluted before injection. The saponin was dissolved in normal saline.

Animals and sites of inoculation.—Young adult albino and himalayan rabbits were used; they were injected in the lateral ear vein. The guinea-pigs were stock adults; injections were given into the saphenous vein after its exposure through a small skin incision. The mice were from a closely inbred laboratory strain: inoculations (never exceeding 0·45 ml.) were made into the dorsal tail vein.

Methods used in attempts to dissociate the lethal and haemolytic actions of streptolysin O.—(a) *Heat:* The streptolysin solution was heated at 56° for 1 hr. (b) *Inhibition by cholesterol:* Equal volumes of a saturated solution of cholesterol in ligroin and streptolysin solution were shaken together in a separating funnel: the lower lysin layer was then run out. (c)

Tryptic digestion : 4·8 ml. streptolysin solution and 0·2 ml. filtered solution of trypsin (B.D.H.) (1 per cent in 0·07 M phosphate buffer pH 7·6) were mixed and left at room temperature for 6 hr. (d) *Neutralization by antistreptolysin :* 4·8 ml. streptolysin solution and 0·2 ml. streptolysin O standard antitoxin were mixed and left at room temperature for 15 min. before use. (e) *Dialysis :* The streptolysin solution was dialysed in a cellophane sac overnight in the cold against distilled water and then re-concentrated to volume in the same sac in a current of air.

In all the above experiments the residual haemolytic and lethal potencies were evaluated in terms of the untreated streptolysin solution. In each instance the streptolysin O was activated with sodium thioglycollate before treatment.

Controls.—(a) *Sodium thioglycollate solution :* Control animals of each species were injected with this solution in the same amounts as those that accompanied the streptolysin ; no evidence of intoxication was seen. (b) *Neutralization of streptolysin by ligroin :* Streptolysin solution was shaken with an equal volume of ligroin in a separating funnel, and the lysin run out as before ; no loss of haemolytic activity was found. (c) *Toxic effects of the concentrated medium :* To exclude the possibility that the observed toxic effects might be due to either the medium or its concentrates, animals were injected with mixtures of these solutions and streptolysin O standard antitoxin ; such animals showed no signs of intoxication.

Production of hypercholesterolaemia in rabbits.—Following the method of Kellner, Correll and Ladd (1951) and Cornforth, Hart, Rees and Stock (1951), 6 rabbits were each given 4 intravenous injections of 12·5 per cent aqueous solution of " Triton WR 1339 " (4 ml./kg. body wt.) at intervals of 3, 2, 2 days. Blood cholesterol was estimated just before each injection by the method described by King (1951). One rabbit died on the 8th day, but the rest continued healthy throughout. On the 11th day blood was removed for cholesterol estimation and streptolysin injected immediately afterwards. The initial cholesterol values ranged from 60 to 130 (mean 100) mg./100 ml., and rose to from 925 to 1350 (mean 1075) mg./100 ml. by the 11th day.

RESULTS.

In Table I are shown the effects of various doses of streptolysin O and saponin in rabbits, guinea-pigs and mice. The outstanding feature of these results is that the lethal dose of the former (expressed in terms of our previously-defined rabbit haemolytic unit (H.U.) per g. body wt.) is about 30 times greater for mice than for either of the other species. With saponin, on the other hand, the lethal doses for all three species are closely the same. It may be noted that these results closely parallel the comparative haemolytic potencies of these lysins for these species recorded in the previous paper.

The results in Table II show that when streptolysin O was subjected to procedures calculated to act detrimentally on its specific activities, no evidence of dissociation was found. Whether neutralized by specific standard antitoxin, absorbed with cholesterol, digested with trypsin or heated at 56°, the streptolysin lost its ability both to lyse red cells and to kill mice. Conversely, when exposed to dialysis and subsequently re-concentrated to volume, it lost neither haemolytic nor lethal properties.

The effect of streptolysin on rabbits rendered hypercholesterolaemic with " Triton WR 1339 " injections is shown in Table III. Despite the high blood cholesterol levels (roughly 10 times normal) at the time streptolysin was injected there was no increased resistance to the lethal action of the toxin.

DISCUSSION.

When streptolysin O is given intravenously to rabbits, guinea-pigs and mice, the minimal lethal dose per unit of body weight is found to be much larger for the mouse than for either of the other species, a difference paralleled by the exceptional

resistance of mouse cells to this lysin *in vitro* (Howard and Wallace, 1953). Although the potency of our streptolysin preparation cannot be evaluated closely, it is apparent that while the m.l.d. for rabbits and guinea-pigs are both about

TABLE I.—*The Effects of Intravenous Injections of Various Doses of Streptolysin O and Saponin on Rabbits, Guinea-pigs and Mice.*

Dose (H.U./g.).	Streptolysin O.			Saponin.		
	Rabbits.	Guinea-pigs.	Mice.	Rabbits.	Guinea-pigs.	Mice.
7·0	—	—	D* 9/9† ($\frac{1}{2}$, $1\frac{1}{2}$, $1\frac{1}{2}$ 2, 2, 3, 3, 4 min. and 8 hr.)	—	—	—
6·0	—	—	D 3/3 (2, 5 and 25 min.)	—	—	—
5·0	—	—	D 1/2 (1 hr.)	—	—	—
4·0–2·0	—	—	S 6/6	—	—	—
0·60	—	D 2/2 (6 and 18 hr.)	—	—	—	D 3/3 ($2\frac{1}{4}$, 3 and 5 hr.).
0·50	—	—	—	—	D 1/1 ($2\frac{1}{2}$ hr.)	—
0·45	—	D 1/1 (24 hr.)	—	—	—	—
0·40	—	—	—	—	D 1/1 (3 hr.)	D 3/3 ($3\frac{1}{4}$, $3\frac{1}{4}$ and $3\frac{1}{2}$ hr.).
0·30	D 2/2 (2 and 12 hr.)	D 1/1 (48 hr.)	—	D 1/1 (12 hr.)	D 1/1 ($2\frac{1}{2}$ hr.)	—
0·25	D 1/1 (9 hr.)	D 2/2 (18 and 38 hr.)	—	—	S 1/1	—
0·20	D 1/2 (12 hr.)	D 1/2 (15 hr.)	—	—	D 1/1 (3 hr.)	D 3/3 ($3\frac{3}{4}$, 5 and 18 hr.).
0·15	D 1/1 (12 hr.)	S 1/1	—	D 1/1 (29 hr.)	S 1/1	D 3/3 (22, 72 and 72 hr.).
0·12	—	—	—	—	—	D 2/3 (22 and 22 hr.).
0·10	D 1/2 (12 hr.)	S 1/1	—	S 1/1	S 1/1	D 2/6 (18 and 72 hr.).
0·05	S 2/2	—	—	S 1/1	S 1/1	S 1/1

* S and D represent survival or death.
† Numbers of animals tested and times of death in brackets.

0·2 H.U./g. body wt., that for mice is about 6 H.U./g.—a value about 30 times as great. On the other hand, when saponin was used as the toxic and haemolytic agent, no such wide disparity is disclosed, the m.l.d. for rabbits, guinea-pigs and mice being about 0·15, 0·30 and 0·12 H.U./g. respectively. Thus the resistance of the mouse to the lethal action of streptolysin O, like the high resistance of its red cells to lysis by this agent, is outstanding, though its basis remains equally unexplained.

We have failed to corroborate Harris's conclusion that the oxygen-labile haemolysin (streptolysin O) and the lethal factor in unfractionated streptococcal

filtrates are distinct entities which possess recognisably different properties. Repeating the tests he employed, we have failed to confirm his finding that these two factors can be differentiated by heating, cholesterol-absorption, tryptic digestion, dialysis or incubation with a streptolysin O antiserum. The present

TABLE II.—*The Effects of Various Procedures on the Haemolytic and Lethal Properties of Streptolysin O.*

Treatment of streptolysin.	Haemolytic activity.	Dose (H.U./g.).	Lethal activity: number of mice used.	Result.
Addition of antistreptolysin	*nil*	6·5	5	Survival.
Exposure to cholesterol	„	6·5	5	„
„ ligroin only	Present	6·5	2	Death (3 and 3 min.).
„ trypsin	*nil*	6·5	2	Survival.
„ heat (56°, 1 hr.); thioglycollate added—				
(*a*) Before	„	6·5	3	„
(*b*) After	„	6·5	3	„
(*c*) Before and after	„	6·5	3	„
Dialysis	Present	0·6	Guinea-pig (450 g.)	Death (2 hr.).

results accord better with a belief that the haemolytic and lethal actions of these filtrates depend on the same constituent. It may be noted that Bernheimer (1944), working with filtrates from cultures of *Cl. septicum*, found their haemolytic and lethal potencies to agree closely, and concluded that the same agent was probably responsible for both phenomena.

TABLE III.—*The Effect of Streptolysin O on Hypercholesterolaemic Rabbits.*

Blood cholesterol (mg./100 ml.).	Dose (H.U./g.).	Result.
1000	2·0	Death (1 hr.).
925	1·3	„ (1½ hr.).
1050	0·8	„ (12 „).
1075	0·5	„ (22 „).
1350	0·3	„ (12 „).

Although the haemolytic and lethal potencies of streptolysin O and of saponin show a seemingly more than chance parallelism, it would be incorrect to infer that death after small inocula of the former results from any severe grade of haemolysis *in vivo*, or that the survival of mice after large doses of this toxin can be attributed to high resistance of their red cells. Histological sections of the heart, lungs, liver, spleen and kidneys of all three species dying from this intoxication showed some venous congestion but no intravascular haemolysis. This accords with findings of Barnard and Todd (1940). Occasionally, at autopsy, the bladder contained a little blood-stained urine, but in every instance the blood lost was negligible.

In view of the marked affinity of cholesterol for reduced streptolysin O, it seems paradoxical that cholesterol in the plasma of normal animals, and *a fortiori* of the hypercholesterolaemic rabbits treated with "Triton," failed to protect

them against the small inocula of the toxin used. *In vitro* 1 H.U. of our streptolysin O is neutralized by 0·14μg. of emulsified cholesterol, so that a normal rabbit plasma should be able to neutralize about 7000 H.U./ml., and hypercholesterolaemic plasma proportionately more. That plasma cholesterol is for some reason incapable of neutralizing streptolysin O *in vivo* is evident from the fact that a rabbit possessing about 70 ml. plasma succumbs to an inoculum of only about 500 H.U.

The reason for this apparent anomaly is probably traceable to the physicochemical state of the cholesterol in the plasma. The esterified fraction (about two-thirds) cannot neutralize the toxin because esterification involves the hydroxyl group at carbon atom 3, which we have shown (Howard, Wallace and Payling Wright, 1953) to be concerned with the neutralization of the streptolysin. The "free", unesterified cholesterol is probably, as Cohn and Edsall (1949) suggested, in the form of a lipo-protein complex, each protein molecule being associated with about 1000 cholesterol molecules. Blix, Tiselius and Svenssen (1941) showed by electrophoretic separation that most of both esterified and unesterified cholesterol is in complexes with α- and β-globulins, and concluded that this sterol does not occur in the plasma to any significant extent in the unbound state. This inference is supported by Hewitt and Todd's (1939) observation that after treatment with proteolytic enzymes, the ability of rabbit's serum to neutralize streptolysin O rose very greatly, a rise which they attributed to liberation of some previously protein-bound cholesterol.

Because of the lack of protection given by plasma cholesterol, it seems likely that small inocula of streptolysin can make closer contact with the tissue sterols than might be expected. Whether the cholesterol-containing elements in tissue cells are sites for attachment of the streptolysin is uncertain, though its haemolytic action suggests that this might be a stage in the mechanism of cell injury. We have previously shown, however, that streptolysin O has an affinity for some sterols other than cholesterol, and it is possible that *in vivo* the toxin effects damage by forming a complex with one or other of these kindred substances.

SUMMARY.

The lethal effects of streptolysin O and saponin for rabbits, guinea-pigs and mice have been studied. Mice showed much greater resistance to the streptolysin than either of the other species, though the resistance of all three to saponin was closely similar. These findings are in accordance with differences in *in vitro* resistances of red cells of these species to lysis by streptolysin O and by saponin.

The haemolytic and lethal properties of preparations of streptolysin O could not be dissociated by heat treatment, cholesterol absorption, neutralization with antistreptolysin O, trypsinization or dialysis.

Although streptolysin O is rapidly neutralized by cholesterol, rabbits with hypercholesterolaemia produced by "Triton WR 1339" showed no increase in resistance to this toxin. It is suggested that this apparently anomalous finding results from the close binding of almost all this cholesterol to plasma globulins.

Our thanks are due to Dr. L. F. Hewitt of the Serum Laboratories, Carshalton, for a gift of streptolysin O standard antitoxin, and to Messrs. Charles Lennig & Co. (Great Britain) Limited for the "Triton WR 1339."

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OBSERVATIONS ON SCHALES' REACTION FOR HYDROGEN PEROXIDE, WITH SPECIAL REFERENCE TO ITS USE IN COMPLEX SOLUTIONS CONTAINING SUGAR AND PEPTONE.

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In view of investigations carried on for many years in this department on the production of hydrogen peroxide by micro-organisms (McLeod and Gordon, 1922*a*, *b*, 1923*a*, *b*, 1925*a*, *b*; McLeod, Gordon and Pyrah, 1923; Gordon and McLeod, 1928, 1940; McLeod, 1928, 1930*a*, *b*), we noted with particular interest the reference by Jordan (1952*a*, *b*) to the use of Schales' reagent in demonstrating hydrogen peroxide production by *P. septica*.

Through the kindness of Miss Jordan a culture of the strain used in her observations was obtained, and this, as expected, gave positive catalase and oxydase reactions, and did not produce green discoloration when growing on heated blood agar or blackening on benzidine blood agar as other peroxide-producing bacteria do, even when it is very difficult to demonstrate H_2O_2 in their cultures in fluid media.

The Schales' reagent used, however, is more delicate than many others, and it was only with this test that Jordan describes positive results. If these results are reliable, therefore, it is clearly a reagent of special value. It is nevertheless obvious that any results obtained with a reagent of peculiar delicacy must be subjected to elaborate and rigid control before they can be accepted.

EXPERIMENTAL.

In the first place it was established that a solution of H_2O_2 of standardised strength still gave a faint positive test with freshly prepared Schales' reagent when diluted to a concentration of 10^{-7}, whereas 10^{-6} was the limit for the well established test with Schönbein's reagent (starch and cadmium iodide with a trace of $FeSO_4$ as catalyst). In this respect, as anticipated, our results correspond with those of Schales (1938) and other observers.

The next obvious line of investigation was to study a variety of substances for their capacity to enhance or diminish the sensitivity of this reagent. Sugars, amino-acids, peptides and peptones are amongst the substances most commonly present in bacteriological media, and some of these were therefore examined.

Effect of sugars.

In her second paper Jordan (1952*b*) describes the production of varying amounts of H_2O_2 when sediments of *P. septica* grown on a complex but defined medium, are shaken with different sugar solutions and other substrates in a Warburg respiration vessel. Using lactic acid as substrate no H_2O_2 was detected, whereas

with fructose and sucrose concentrations of the order of 10^{-7} were obtained, and with glucose, galactose and maltose even 10^{-5}. These variations in peroxide formation were not, however, related in any definite way to the amount of O_2 absorbed.

Our first observations on the influence of glucose solutions on the colour developed by Schales' reagent in contact with dilute peroxide solutions were difficult to read on account of the appearance of colour in controls which only contained glucose and the reagent. This led us to test dilutions of different sugars with Schales' reagent. The results are given in Table I.

TABLE I.—*Tests of Sugar Solutions with Schales' Reagent.*

Sugar.	Concentration per cent.	Result obtained with Schales' reagent.	Approx. conc. of H_2O_2 developed.
Glucose	5 to 0·003	All positive (0·1 per cent gave deepest colour)	10^{-4}–10^{-6}
Lactose	0·1	Positive	10^{-6}
Maltose	0·1	"	10^{-6}
Galactose	0·1	Slightly positive	10^{-7}
Fructose	0·1	" "	10^{-7}
Sucrose	0·1	Trace	10^{-8}

These results resemble closely those obtained in the presence of *P. septica* by Jordan. They suggest, at least, extreme caution in interpreting reactions with Schales' reagent carried out in the presence of glucose ; *i.e.*, it must be established that these results are not produced from the interaction of the reagent with the sugar.

This reagent consists of phenolphthalein in a concentrated solution of NaOH. It is well known that in the presence of NaOH, glucose is partly decomposed on heating, in such a way as to effect rapid reduction of methylene blue. (A solution of methylene blue reduced in this way is the most commonly used indicator for controlling the exclusion of oxygen from anaerobic vessels.) Apparently there is a production of active H_2 donators which in the presence of O_2 are likely to reduce that gas to H_2O_2. Further evidence in support of this conclusion is provided by observations on the interaction of Schales' reagent and glucose solution in the absence of oxygen. If sugar solution and Schales' reagent in appropriate amounts are disposed in two limbs of a Y tube, and the whole is evacuated, filled with N_2 and sealed, the sugar and reagents when mixed will remain colourless for days, but develop a pink colour shortly after the admission of air. It was observed further that the other sugars mentioned above which gave positive reactions with Schales' reagent, can be substituted effectively for glucose in the reduction of methylene blue for preparing indicators of the absence of O_2 in anaerobic apparatus, with the exception of sucrose.

In view of all this, it seems altogether probable that traces of H_2O_2 are produced in glucose solutions when in contact with Schales' reagent under aerobic conditions, and hence no deduction of H_2O_2 production under the influence of bacterial sediments can safely be made in these circumstances unless elaborate controls, of which there is no mention in the papers referred to, are adopted. It was observed further that the H_2O_2 reaction with Schales' reagent in the presence of glucose was obliterated when 1 per cent peptone solution was also present. The same result was obtained with several different peptones. This,

however, is not surprising, as peptones are equally effective in obliterating positive H_2O_2 reactions with other reagents, provided that the H_2O_2 concentrations are of the order of 10^{-6}.

Effect of protein derivatives.

In the long-established biuret reaction for peptone and other derivatives of protein digestion a gradation of colours from violet to pink is obtained, varying with the concentration and phase of degradation of the soluble protein substance present. The standard text-book instructions for carrying out such a reaction require the addition of a solution of $CuSO_4$ and NaOH to the fluid under investigation. The concentration of Cu and of NaOH recommended vary.

Gies' reagent is defined by Hawk (1923) as 25 ml. of 3 per cent $CuSO_4$ per l. 10 per cent NaOH. Both the alkali and Cu required for such tests are present in Schales' reagent, and to detect whether positive tests for protein derivatives might be a further source of confusion in using this reagent to detect traces of peroxide the tests were made which are detailed in Table II.

TABLE II.—*Effect of Varying Concentrations of Copper on Biuret Reaction.*

Amounts of $CuSO_4$.	Peptone 1 per cent (ml.).	N NaOH (ml.).	Result.
0·01N 1 drop	1	0·1	No colour
„ 2 „	1	0·1	„
„ 3 „	1	0·1	„
„ 4 „	1	0·1	Trace pink
„ 5 „	1	0·1	Increasing
„ 6 „	1	0·1	intensity
„ 7 „	1	0·1	of
„ 8 „	1	0·1	pink
„ 9 „	1	0·1	colour
0·1N 1 „	1	0·1	pink colour = 10^{-6} H_2O_2 with Schales' reagent

From these results it is clear that in using the reagent in the way recommended by Schales there is no danger of error due to the presence of small amounts of protein derivatives, but that such sources of confusion do enter as soon as the Cu content of the reagent is increased 10-fold as recommended by Jordan (1952*a*) in testing for H_2O_2 in the presence of bacteria.

How far traces of proteins and protein degradation products may be present in suspensions of "resting bacteria" from complex defined media cannot be assessed theoretically, but if present in any considerable amount they might well lead to false peroxide reactions with Schales' reagent. In any case it seems not improbable that a biuret reaction is responsible for the statement (Jordan, 1952*a*) that T.M.B. medium (Fildes and McIntosh' modification of Douglas' tryptic digest of meat), containing 4 per cent soluble solids and 0·7 per cent amino N, gave a Schales' reaction indicating the presence of 0·1 μg. H_2O_2/ml., assuming that the test was carried out with a 10-fold increase of Cu in Schales' reagent.

A considerable amount of valuable indirect evidence for peroxide production by *P. septica* is brought forward in Jordan's papers, but in view of the above findings her direct evidence of peroxide production based on Schales' reaction and the figures for concentrations developed must be accepted with reserve.

There is not yet therefore any satisfactory evidence that catalase-active bacteria produce concentrations of peroxide titratable by recognised chemical reagents for that substance.

SUMMARY.

Schales' reagent, although undoubtedly capable of detecting minute traces of H_2O_2, must be used with great caution in the presence of sugar solutions, since the strong alkali contained in the reagent tends to produce H_2-donating substances from the sugar which will release traces of peroxide under aerobic conditions.

If the Cu content recommended in the original description of the reagent is exceeded biuret protein reactions may be a source of confusion.

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THE EFFECT OF SOME ANTIBIOTICS ON COCCAL INFECTION IN MICE.

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In certain circumstances penicillin and terramycin can be completely bactericidal *in vivo*, for in rabbits made granulopenic with benzene large systemic doses of either drug will sterilise the leucocyte-free skin lesions produced by pneumococci (Gowans, 1953). However, it is possible that if the treatment given to an infected normal animal is by itself insufficient to kill all the bacteria, complete sterilisation may yet be possible with the assistance of phagocytes. Jawetz (1946) and Eagle, Fleischman and Musselman (1950) have suggested that penicillin may render bacteria more susceptible to the natural body defences.

In the work reported here the relation of phagocytosis to the fate of infecting bacteria was investigated by examining the peritoneal exudate from normal and leucopenic mice infected intraperitoneally with virulent pyogenic cocci and treated systemically with single doses or short courses of antibiotics.

METHODS.

Female mice of about 20 g. were infected intraperitoneally with a dilution of serum broth cultures of virulent organisms. Infected mice were divided into four groups: one control and one treated group were allowed to survive to check the virulence and determine the degree of protection afforded by treatment; pairs of mice from the other control and treated groups were killed at intervals from the start of treatment. Swabs from the peritoneal cavity of each mouse were cultivated on blood agar plates, and films of the exudate were stained with Gram's and Wright's stains. Films were made by gently drawing a slide over the surface of the intestines or, if the exudate was very copious, by collecting it in a fine pipette and spreading on a slide by the usual method for making blood films. When the number of colonies in the cultures was small it was recorded exactly; more abundant growth was recorded arbitrarily as " scattered colonies " (about 500–1000 colonies), " numerous discrete " (more than 1000 discrete colonies), or " confluent."

Leucopenia was produced by a single subcutaneous injection of 3 mg. 1 : 4-dimethanesulphonoxybutane in 0·2 ml. arachis oil, which almost eliminated granulocytes from the blood in a high proportion of mice but left them well enough for a therapeutic experiment. The leucocyte counts of 10 mice, 7 days after an injection, are given in the Table. The drug, which is extremely toxic, has been used for the suppression of tumours in experimental animals and in a therapeutic trial for myeloid leukaemia in man (Haddow, 1950). It seemed to have no significant antibacterial activity of its own since streptococci in the peritoneum of leucopenic mice grew exuberantly.

RESULTS.

Infection in Normal Mice.

Pneumococcal infection treated with penicillin or terramycin.

Mice were infected with 0·2 ml. of a 1/1000 dilution of a 6 hr. culture of virulent Type 1 pneumococcus (Wellcome CN 33) which had a lethal dose of less than

50 organisms. Twelve hr. after infection they were treated with either a single dose of 200 units penicillin G subcutaneously, or with 1 mg. terramycin hydrochloride intravenously (commercial preparation buffered with sodium glycinate). Untreated mice died within 36 hr., 10/10 receiving penicillin died between 48 and 72 hr., and 9/10 receiving terramycin died between 70 and 95 hr. after infection.

In all cases peritoneal swabs at the start of treatment gave confluent growth, but by 3 hr. it was reduced to scattered colonies; the exudate from a mouse treated 9 hr. previously with terramycin grew 160 colonies after prolonged incubation. With penicillin surviving organisms began to multiply after 6 hr. but with terramycin not until 24 hr.; thereafter growth rapidly became confluent in both groups.

With either drug the number of extracellular cocci was greatly reduced in 3 hr. although there was almost no phagocytosis by granulocytes; many organisms became Gram-negative. Pneumococci surviving terramycin treatment were pleomorphic and stained deeply and by 6 hr. were taken up by macrophages which appeared in large numbers, but by 24 hr. the macrophages were degenerating and swollen extracellular cocci were again present (Fig. 1, 2, 3, 4, 5). With

DESCRIPTION OF PLATES.

All figures × 10 eyepiece, 2 mm. objective.

FIG. 1–5.—Films of exudate from mice infected intraperitoneally with pneumococci and treated 12 hr. later with a single intravenous dose of 1 mg. terramycin.

FIG. 1.—Start of treatment. Numerous extracellular cocci and no phagocytosis by granulocytes.

FIG. 2.—Three hr. after treatment. A decrease in the number of extracellular cocci has occurred and the surviving organisms are swollen.

FIG. 3.—Six hr. after treatment. Macrophages containing diplococci were numerous at this time.

FIG. 4.—Twenty-four hr. after treatment. The exudate contains extracellular swollen cocci and the cells are degenerating.

FIG. 5.—Forty-eight hr. after treatment. The exudate is overgrown with pneumococci.

FIG. 6.—Peritoneal exudate from a mouse 24 hr. after infection with *Staph. aureus*. Treatment with 5 mg. cephalosporin intravenously was given 1, 7 and 11 hr. after infection. The granulocyte in the upper left-hand corner contains staphylococci and is degenerating; below there is a macrophage containing many cocci.

FIG. 7.—Exudate from a mouse 31 hr. after infection with *Staph. aureus*. Treatment with three subcutaneous doses of 0·5 mg. penicillin was given 1, 7 and 11 hr. after infection. The macrophages appear to have ruptured and are liberating large numbers of cocci.

FIG. 8 and 9.—Exudate from mice infected with *Str. pyogenes* and treated with an intravenous dose of 0·5 mg. micrococcin-Triton 12 and 18 hr. after infection.

FIG. 8.—Three hr. after the start of treatment. There are many extracellular cocci and marked phagocytosis by granulocytes.

FIG. 9.—Twenty-four hr. after the start of treatment. The cocci are undergoing digestion.

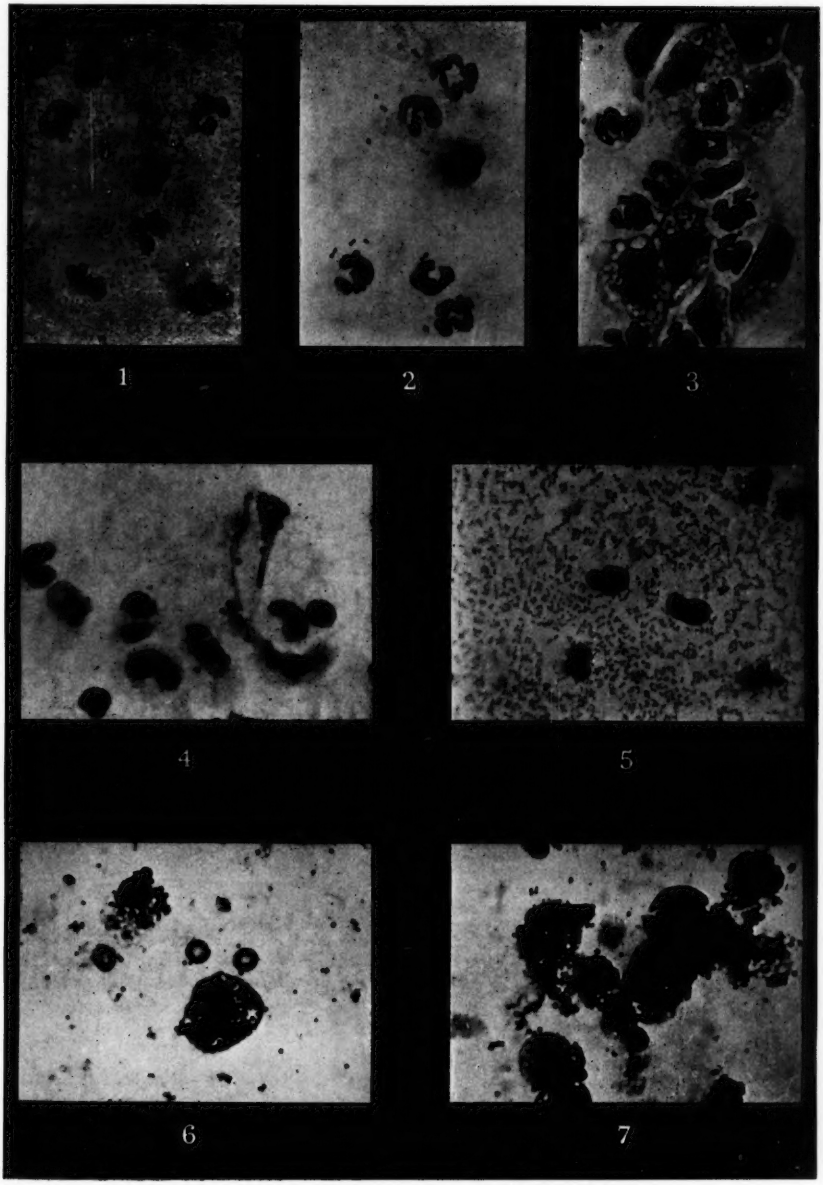
FIG. 10–17.—Exudate from normal and leucopenic mice 13 hr. after intraperitoneal infection with *Str. pyogenes*. Treatment with a single intravenous dose of antibiotic was given 1 hr. after infection. Fig. 10–13, normal mice; fig. 14–17, leucopenic mice. Exudates from leucopenic mice contained more viable cocci than those from normal mice (see Table). Except in the case of penicillin, exudates from leucopenic mice show more extracellular cocci than those from normal mice.

FIG. 10 and 14.—Treated with 0·5 mg. penicillin.

FIG. 11 and 15.—Treated with 1 mg. micrococcin-Triton.

FIG. 12 and 16.—Treated with 0·5 mg. terramycin.

FIG. 13 and 17.—Treated with 0·5 mg. aureomycin.



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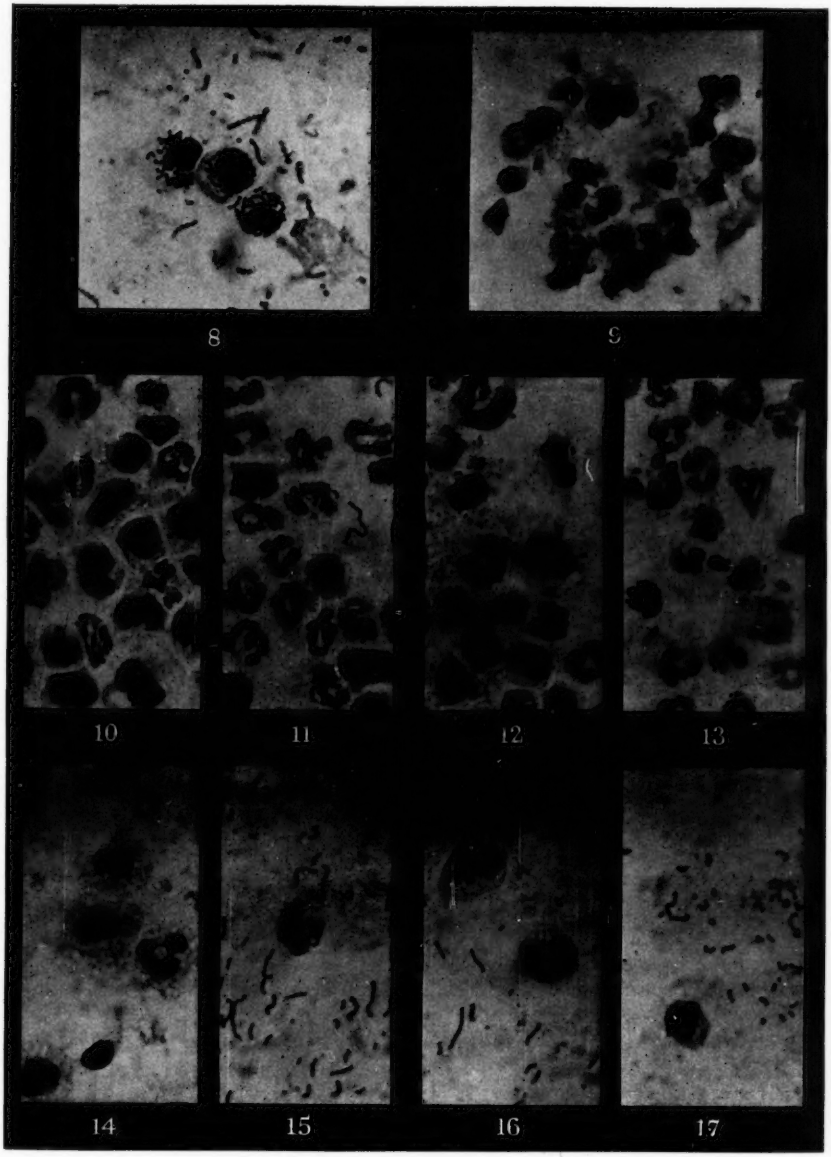

Gowans.

penicillin, macrophages were much less numerous and some extracellular cocci were always present.

Thus, penicillin and terramycin were both rapidly bactericidal. Granulocytes did not appear to share in the removal of organisms, but in the terramycin group surviving cocci were taken up by macrophages. In neither group were all the organisms eliminated and those that remained grew and killed the animals.

Streptococcal infection treated with penicillin or micrococcin-Triton.

Micrococcin-Triton (Heatley, Gowans, Florey and Sanders, 1952) is very active against *Str. pyogenes in vitro* but is bacteriostatic even at high concentrations and relatively ineffective in experimental infections. In the present experiments its action *in vivo* was compared with that of a bactericidal agent, penicillin.

Mice were infected with 0·2 ml. of a 1/10,000 dilution of a 6 hr. culture of *Str. pyogenes* (Wellcome CN 10), which had a lethal dose of about 50 organisms. They were treated with either a single dose of 200 units penicillin G subcutaneously 12 hr. after infection or 0·5 mg. micrococcin-Triton intravenously at 12 and at 18 hr. Untreated mice died within 36 hr., 9/10 receiving penicillin died between 48 and 96 hr., and 8/10 receiving micrococcin-Triton died between 48 and 168 hr. after infection.

Peritoneal swabs at the start of treatment gave confluent growth. Penicillin reduced this to scattered colonies in 3 hr., but a rapid re-growth took place after 10 hr. In contrast, with micrococcin-Triton there was no reduction in growth until 24 hr. after starting treatment, and at 32 hr. the exudate was sterile in the two mice examined. However, mice relapsing after micrococcin-Triton gave confluent cultures.

At the onset of treatment there were many extracellular cocci and some phagocytosis by the numerous granulocytes. Penicillin rapidly reduced the number of extracellular cocci; by 3 hr. most of them had been ingested by granulocytes aggregated into foci on the peritoneal surface, but by 24 hr. regrowth of surviving organisms had occurred and extracellular chains of cocci were numerous. Three hr. after starting treatment with micrococcin-Triton there was very active phagocytosis by granulocytes (Fig. 8). The number of extracellular cocci declined slowly and those in the aggregations of granulocytes were slowly digested (Fig. 9), until at 32 hr. there were no extra-cellular cocci and the intracellular organisms appeared to be undergoing digestion in granulocytes and macrophages. Nevertheless the majority of micrococcin-Triton-treated mice eventually died with the peritoneum crowded with cocci; evidently a few cocci survived and later multiplied.

Staphylococcal infection treated with penicillin or cephalosporin.

Cephalosporin (Ritchie, Smith and Florey, 1951) is very active against staphylococci *in vitro* though it fails to control infection in mice satisfactorily. Unpublished data (Smith, 1952) suggest that the discrepancy is due to the inactivation of cephalosporin by serum.

Mice were infected with 0·5 ml. of a 24 hr. culture of *Staph. aureus* (CN 491) diluted 1/10,000 in 7 per cent Wilson's gastric mucin; 0·5 ml. of a 10^{-7} dilution was lethal to mice. At 1, 6 and 10 hr. after infection mice were given either

0·5 mg. penicillin subcutaneously or 5 mg. cephalosporin intravenously. Untreated mice died within 24 hr., 8/10 receiving penicillin died between 45 and 95 hr. and 10/10 receiving cephalosporin died between 27 and 82 hr. after infection.

At the start of treatment swabs of exudate grew about 600 colonies. Penicillin reduced growth to 47 and 5 colonies at 3 and 6 hr. respectively; growth had re-started by 10 hr. and cultures were confluent by 23 hr. Cephalosporin did not reduce the number of viable organisms and, as with untreated mice, the cultures were confluent 7 hr. after infection.

Six hr. after starting penicillin treatment there were no extra-cellular cocci and only a few swollen cocci within granulocytes. By 23 hr. many macrophages had appeared and there were numerous intracellular organisms in granulocytes and in macrophages, some of which contained 30–40 cocci. At 30 hr. degenerating macrophages appeared to be liberating large numbers of cocci (Fig. 7). With cephalosporin the majority of organisms were still intra-cellular in granulocytes at 10 hr., many being pleomorphic. By 23 hr. granulocytes were degenerating, there were many extracellular cocci and many macrophages were filled with cocci (Fig. 6).

In this experiment the relative inactivity of cephalosporin against staphylococcal infection was confirmed, but since 1·5 mg. penicillin did not cure all the similarly infected mice the test was a rigorous one.

Streptococcal Infection in Normal and Leucopenic Mice.

Micrococcin-Triton is only bacteriostatic *in vitro*, yet treatment of streptococcal infection eliminated almost all the organisms from the peritoneum. Phagocytosis and intracellular digestion of streptococci were prominent, and it seemed probable that phagocytes had assisted the drug to kill the organisms. However, stained films do not show whether phagocytes augment the action of antibiotics or merely remove already killed bacteria. This was investigated by comparing the ability of normal and leucopenic mice to destroy streptococci after intravenous injection of antibiotics.

Ten normal and ten leucopenic mice were infected with 0·2 ml. of a 1/10 dilution of a 2 hr. culture of *Str. pyogenes* grown from a large inoculum. One hr. later a single dose of either micrococcin-Triton, penicillin, terramycin or aureomycin was given intravenously, each drug being given to two normal and two leucopenic mice. Two infected pairs received no treatment. All mice were killed 12 hr. after treatment and the peritoneal exudate and heart blood examined. Four groups of three normal mice were also infected and treated, but allowed to survive to show the protection afforded by the treatment. All these normal mice eventually died: those treated with micrococcin-Triton between 3 and 6 days after infection, with penicillin between 3 and 10 days, with terramycin between 1 and 5 days, and with aureomycin between 2 and 7 days. Untreated normal mice were dead by 27 hr. and untreated leucopenic mice by 21 hr.

The Table summarises the experiment and shows the growth obtained from cultures of heart blood and peritoneal exudate. Twelve hr. after treatment there were consistently fewer viable organisms in the blood and exudate of normal than of leucopenic mice, especially in those treated with penicillin and aureomycin.

TABLE.—*Growth from Blood and Peritoneal Exudate of Normal and Leucopenic Mice Infected Intraperitoneally with* Str. pyogenes *and Treated One Hour Later with Single Intravenous Injections of Antibiotics.*

Antibiotic.	Intravenous dose in mg.	Condition of mice.*	Number of colonies on blood-agar plates from		Leucocyte counts of leucopenic mice at time of infection. Cells per c.mm.†	
			Swab of peritoneal cavity.	Heart blood.	Total.	Polymorphs.
Micrococcin-Triton	1·0	Normal	Numerous discrete	7		
		Leucopenic	Confluent	Numerous discrete	5050	70
					4780	70
Penicillin	0·5	Normal	1	7		
		Leucopenic	Numerous discrete	480	1210	20
					3350	20
Terramycin	0·5	Normal	Numerous discrete	6		
		Leucopenic	Confluent	90	3840	40
					3160	70
Aureomycin	0·5	Normal	35	Sterile		
		Leucopenic	Confluent	530	2090	0
					3720	0
None	—	Normal	„	Confluent		
		Leucopenic	„	„	6050	300
					2860	110

* Two normal and two leucopenic mice were used in each experiment. Exudate from the first mouse of each pair was used for bacteriological study, that from the second mouse for the preparation of stained films. All samples taken 13 hr. after infection.

† Figures given for both mice of each leucopenic pair.

In comparison with normal mice the exudates from leucopenic mice showed very few cells and most of these were macrophages. Exudates from treated leucopenic mice showed many extracellular cocci, with the exception of those treated with penicillin, in which extracellular organisms were hard to find although there were many single cocci in macrophages (Fig. 14–17). Exudates from normal treated mice showed many fewer cocci, most of them intracellular (Fig. 10–13).

Phagocytosis by granulocytes therefore appeared to assist the antibiotics to remove and kill streptococci. Nevertheless all the normal treated mice relapsed and died despite the very cellular exudates and the few cocci in them 12 hr. after treatment.

DISCUSSION.

In antibiotic-treated mice phagocytosis by granulocytes played an important part in eliminating streptococci, though not pneumococci. The rapid disappearance of extracellular pneumococci was probably due to lysis. Streptococci, however, are not readily lysed (Todd, 1945), and in mice treated with penicillin or micrococcin-Triton they were removed by intracellular digestion in groups of phagocytes adhering to the peritoneum. The experiments with leucopenic mice suggest that granulocytes actually assist antibiotics to kill streptococci and do not merely remove dead organisms.

The difference in action between micrococcin-Triton which is bacteriostatic *in vitro* and penicillin or terramycin which are bactericidal was reflected in the

speed with which viable cocci were cleared from the peritoneum. Penicillin or terramycin greatly reduced the numbers in 3 hr., but with micrococcin-Triton the number of viable streptococci did not fall until 24 hr. after starting treatment. Micrococcin-Triton persists in the animal for a considerable time after intravenous injection (Heatley *et al.*, 1952), and it presumably prevented multiplication of cocci and allowed phagocytes to destroy them. The majority of streptococci in a large inoculum was killed, but the very few remaining organisms that resisted the cellular defences retained virulence and caused relapse and death.

Eagle *et al.* (1950) found that pneumococci surviving in mice after treatment with penicillin took several hours to resume growth but had not lost virulence. The findings with terramycin in these experiments were similar; a few swollen deeply staining pneumococci remained after the initial rapid bactericidal action and grew slowly when isolated on blood-agar; they caused relapse despite extensive phagocytosis by macrophages. Another example of the resistance of surviving organisms to destruction by phagocytes is provided by the response of staphylococcal infection to penicillin. In one case peritoneal culture grew only five colonies and smears showed many morphologically normal leucocytes containing a few cocci, yet nearly all such mice relapsed; normally, millions of staphylococci in a broth culture must be injected to produce an acute fatal infection. The use of mucin as a suspending agent for the inoculum may have influenced the survival of the staphylococci, for MacCabe and King (1951) have shown that a fraction from mucin damages leucocytes and inhibits phagocytosis, but no evidence of this phenomenon was seen. In mice treated with cephalosporin, or inadequately with penicillin, staphylococci appeared to multiply inside macrophages and destroy them, a finding similar to the *in vitro* observation of Rogers and Tompsett (1952) that virulent staphylococci will multiply inside granulocytes and destroy them while avirulent strains are digested.

It was not expected that the short courses of antibiotics given to these heavily infected mice would be curative, yet in many mice treatment did almost eliminate viable organisms. Despite this, similarly treated mice that were allowed to survive nearly always relapsed and died. With micrococcin-Triton, drug-resistant streptococci were probably responsible for many of these relapses (Heatley *et al.*, 1952), but the nature of the cocci surviving treatment with other antibiotics is not clear. They may possibly correspond to the "persisters" that have been detected *in vitro* (Bigger, 1944; Spicer and Blitz, 1948).

These experiments suggest that to be certain of curing acute infections with virulent organisms, antibiotics by themselves must exert a very effective bactericidal action *in vivo*.

SUMMARY.

Mice were infected intraperitoneally with pyogenic cocci and treated with antibiotics; bacteriological and cytological studies were made of the peritoneal exudates to determine the fate of the bacteria and the part played by phagocytes in removing them.

Penicillin, micrococcin-Triton, aureomycin and terramycin killed fewer streptococci in leucopenic than in normal mice, yet normal mice infected with streptococci, pneumococci or staphylococci commonly relapsed even when the number of organisms surviving treatment was very small.

These experiments were carried out under the direction of Professor Sir Howard Florey, F.R.S., to whom I am indebted for advice and encouragement. My thanks are due to Mr. J. Kent and Mr. I. Chinn for valuable technical assistance, and to Dr. G. M. Watson, Dr. M. A. Jennings and Miss D. Collett for help in the preparation of this paper. The dimethanesulphonoxybutane was kindly supplied by Professor Haddow and the aureomycin and terramycin for intravenous administration by Lederle & Co. and Pfizer & Co. respectively. The work was carried out during the tenure of a Medical Research Council Studentship for training in research methods.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

1. Production, Purification and Properties of the Toxin.

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The literature of the neurotoxin of *Shigella shigae* has recently been reviewed by Engley (1952), and it remains only to summarise those observations that are immediately relevant to this paper. Rough strains of *Sh. shigae* contain a neurotoxic protein which is often referred to as a "true" toxin or an "exotoxin," because it may be liberated in the medium on autolysis of the organism and because it is neutralised by antitoxin in stoicheiometric proportions. It is particularly toxic to rabbits and produces paralysis. Smooth strains of the organism contain both this neurotoxin and a considerably less potent polymolecular enterotoxic complex of phospholipid, polysaccharide and protein which is identical with the dominant O somatic antigen. The existence of two toxins has been questioned from time to time but there remains little doubt that they both exist.

Most of the literature dealing with the neurotoxin refers to crude preparations and few attempts have been made to purify it. Anderson, Brown and Macsween (1945) obtained preparations containing 44–87 Lf/mg. and about 2 LD_{50}/mg. per kg. mouse. Dubos and Geiger (1946), who showed that the presence of iron in the culture medium inhibited the production of toxin, obtained a preparation containing 20 LD_{50}/mg. per kg. mouse and 1500–3000 LD_{50}/mg. per kg. rabbit. These preparations, however, could not have contained more than 1–4 per cent of the toxin, since this paper describes a preparation containing 750 LD_{50}/mg. per kg. mouse, 1,150,000 LD_{50}/mg. per kg. rabbit, and 5,500 Lf/mg. Engley (1952) mentions, but does not describe, preparations containing about 200 LD_{50}/mg. per kg. mouse and 30,000 LD_{50}/mg. per kg. rabbit. Shiga neurotoxin is therefore one of the most toxic substances known, being as toxic as tetanus and botulinus neurotoxins. On the other hand, it occurs in extremely low concentration in cultures of the organism, and consequently its purification presented some difficult problems.

METHODS.

The Organism.

The work was carried out with the rough strain of *Sh. shigae* used by Dubos and Geiger (1946) and kindly supplied by Dr. R. J. Dubos. It gave large flat rough colonies on the surface of nutrient agar and formed almost completely stable suspensions in 0·5 per cent saline. It was partially agglutinated by normal rabbit serum at a dilution of 1/50, but with specific O antiserum (titre 1/1600) at dilutions of 1/100–1/1600 the agglutination observed was no more than that in saline controls. Absorption of this serum at 1/100 with 10^{10} organisms/ml. failed to reduce the titre for the homologous smooth organism, showing that O antigen was absent.

At every harvesting a check was kept on the purity of the culture by microscopic examination and plating. On some occasions it was tested by fermentation reactions which were typical for *Sh. shigae*.

In order to obtain as uniform an inoculum as possible throughout the work, the growth from an overnight culture on the surface of nutrient agar was lyophilised by Stamp's (1947) method in a series of tubes. For use, one tube was opened and subcultivated on casein hydrolysate yeast agar (Gladstone and Fildes, 1940), allowed to grow overnight and then kept in the refrigerator. From this master culture, which was renewed every 7–10 days, a fresh 18-hour subculture was made for each experiment.

The Medium.

It was found that the CCY broth of Gladstone and Fildes (1940) supported vigorous growth of the organism, and produced high yields of toxin when iron was removed by adsorption on calcium phosphate in the manner described in another paper (van Heyningen and Gladstone, 1953). This resulted in a medium containing about 0·05 μg. Fe/ml. and an insufficient level of magnesium to support optimal growth: 20 μg. Mg/ml. and 0·1 μg. Fe/ml. were then added to the medium. The level of 0·15 μg. Fe/ml. is that at which the greatest yield of toxin per unit volume of culture is obtained (about 14 Lf/ml.); below this level a greater yield of toxin per organism is obtained but growth is less.

Cultures.

For cultures on a laboratory scale (1–3 l.) a vertical tubular vessel was used made up mainly of sections of standard "Pyrex" industrial glass pipe line of 2″ (5 cm.) internal diameter (J. A. Jobling & Co., Sunderland). These sections are united by means of standard watertight flanged joints and can be built up into any length, limited only by the size of autoclave necessary to sterilise the apparatus and by ease of handling. The maximum length used was 6 ft. To the lower end of the pipeline a funnel made of a short length of 2″ pipe is joined with the standard flanged joint. Fused across the funnel is a No. 3 sintered glass filter disc, and below this disc the pipe tapers rapidly down to a 0·5 cm. tube attached to an air line. About 0·5 cm. above the disc a short side tube of 0·5 cm. diameter is attached for draining the culture. With the pipeline held vertically and filled to three-fourths of its length with medium, the culture can be very effectively aerated by forcing air through the filter disc. The upper end of the vessel consists of a short section of modified pipeline attached by a flanged joint, and terminating in a dome-shaped glass seal into which are fused 3 glass tubes 0·5 cm. diameter. One of these is attached by rubber tubing to an aspirator containing sterile medium, another to a small funnel for inoculating and for additional constituents of the medium, and the third is an air exit which leads into a trap containing 1 per cent $HgCl_2$ + 0·5 per cent tributyl citrate. The air effluent is finally filtered through a tube 20 cm. long × 2·5 cm. diameter, packed with cotton-wool. The air supply is from a cylinder, and the rate of flow is maintained at 100 ml./min. at 20 cm. Hg pressure. It is sterilised by passage through cotton-wool packed in a tube similar in size to that used for the effluent air, and enters the apparatus at the lower end of the sintered glass funnel through a vertical glass tube at least as tall as the apparatus. Any leakage of culture back through the sintered disc is thus prevented from wetting the cotton-wool filter.

The side arm in the funnel just above the sintered disc is attached to a stop cock through which samples can be withdrawn and the culture harvested.

After smearing the inner wall over the upper quarter with "Silicone" grease to prevent frothing, the empty apparatus is detached from the medium reservoir and effluent trap and sterilised by autoclaving with all flanged joints loosened. The medium, usually 10 l., is autoclaved in the aspirator at 120° for 30 min. The joints in the apparatus are tightened and it is set up in a hot room at 37°. The aspirator is then united with the apparatus with aseptic precautions and a known volume of medium, usually 2 l., as determined by a mark on the outside of the culture vessel, is run in. The culture is inoculated and any further addition to the medium made through the inoculating funnel. After growth the culture is reaped, leaving 50–100 ml., which allows for 2–5 per cent reinoculation when fresh medium is run in. In this way two reapings of 2 l. can be made in 24 hr., and 5 runs made without dismantling. More runs may be made if a fresh reservoir of medium is attached.

Production on a much larger scale was undertaken by Mr. Macsween at the Microbiological Research Department of the Ministry of Supply Station at Porton. The method,

details of which have not yet been published, consisted essentially of the use of a continuous flow of agitated and aerated broth culture which allowed organisms only 1 hr. old to be reaped. Over a course of 7 weeks nearly 2 kg. (dry wt.) of organisms were produced.

Bacterial growth was measured in the way described by van Heyningen and Gladstone (1953).

Assay of Toxin.

The purification of a bacterial toxin is almost impossible unless a rapid and reasonably accurate method of assay is available for testing the numerous fractions prepared. For this reason the Lf was generally determined in routine assays of toxin, since this value could be obtained *in vitro* within 30 min. or less and with greater accuracy than is possible in assays *in vivo*. From time to time the L+ and the LD_{50} were also determined. The Lf was generally equivalent to about 0·7 L+, and about 7 mouse LD_{50} and about 70 rabbit LD_{50}. All dilutions of toxin were made in 1 per cent NaCl containing 0·1 per cent gelatin.

Antitoxin.

The antitoxin used throughout was Anti-Dysentery Serum kindly supplied by Dr. C. L. Oakley of the Wellcome Research Laboratories, Beckenham. It was labelled as containing 50,000 units per 4·5 ml. ampoule and was assumed to contain 11,111 units/ml.

Lf determinations.

For routine Lf determinations seven to ten 0·5 ml. volumes of an appropriate dilution of toxin are pipetted into test-tubes (0·7 cm. internal diameter × 7·5 cm. long), and to these are added falling volumes of a fairly concentrated antitoxin solution with an "Agla" microsyringe. The largest volume of antitoxin is 0·1 ml., the spacing of antitoxin concentration between successive tubes is 10 per cent, and the amount of antitoxin in the middle tube is about 30 units. After mixing the tubes are placed in a water bath at 45° and observed. The water bath is fitted with a glass window in front, and the tubes are illuminated from below by means of a Perspex prism placed in the bath in apposition to a horizontal strip light in front of the window. A black background is placed behind the tubes. The order of flocculation in the tubes can readily be distinguished.

Crude extracts of *Sh. shigae* contain many antigens, and since the antiserum was prepared against crude toxic extracts numerous zones of flocculation may be observed in toxin-antitoxin mixtures. When several 0·5 ml. volumes of a crude preparation of toxin extracted

TABLE I.—*Flocculation in Mixtures of Antitoxin and Crude and Purified Toxin.*

Units antitoxin per ml. mixture.	Flocculation time (min.). Crude pH 11 extract. 17 L+/ml. 25 Lf/ml.		Purified toxin solution. 150 L+/ml. 200Lf/ml.	
1600	—		∞	
800	∞	Antigen I (4000)	∞	
400	10		∞	
200	80	Antigen II (5000)	82	Toxin (1200)
100	50		12	
50	120		45	
25	+	Toxin (875)	∞	
12·5	70		∞	
6·25	120		—	
3·13	+		—	

— = Not observed.
+ = Flocculation overnight.
∞ = No flocculation overnight.

These results show the presence of three flocculating antigens in crude toxin preparations and a single antigen in a purified preparation. Each tube contains 0·5 ml. toxin solution and 0·5 ml. antitoxin solution in falling concentrations. The figures in brackets are the products: units antitoxin per ml. × flocculation time.

from the organisms at pH 11 were mixed with equal volumes of falling 2-fold dilutions of antitoxin and incubated at 45° the tubes containing 400, 100 and 12·5 units of antitoxin per ml. flocculated before any of the others (Table I, col. 2). Since each of these three mixtures was adjacent to mixtures that took longer to flocculate, it was clear that the toxin solution contained at least three flocculating antigens. Assuming that one of these was the toxin, it appeared that the solution contained either *c.* 800, 200 or 25 Lf/ml. Since the solution was found to contain 17 L+/ml. it followed that the true Lf value was more likely to be 25 Lf/ml. than 200 or 800 Lf/ml. When the non-toxic antigens were removed by purification only one flocculation zone appeared (Table I, col. 3). When the toxin was extracted at a higher pH two further non-toxic antigens appeared, as is evident from Table II.

TABLE II.—*Flocculation in Mixtures of Antitoxin and Toxin Extracted at pH* 11 *and* 13.

Units antitoxin per ml. mixture.	Flocculation time (min.) pH 11 extract. 240 L+/ml. 320 Lf/ml.		Flocculation time (min.) pH 13 extract. 200 L+/ml. 256 Lf/ml.	
200	12	Toxin (1020)	∞	
160	7		12	Toxin (896)
128	8		7	
102	12		6	
82	∞		6	Antigen III ? (396)
66	∞		6	
52	∞		5	
42	∞		5	Antigen IV ? (190)
38	∞		5	
26	∞		5	

∞ = No flocculation after 60 min.

Shows appearance of two further non-toxic antigens in pH 13 extract. Each tube contains 0·5 ml. toxin solution and 0·5 ml. antitoxin solution both in falling concentrations.

In this experiment the antitoxin dilutions were closer, *viz.*, 1·25-fold instead of 2-fold, and consequently the different zones are less clearly defined.

In order to distinguish the toxin flocculation zone from the non-toxin zones Anderson *et al.* (1945) did a corresponding L+determination for each Lf determination. This laborious and time-consuming procedure nullifies the advantage of the comparatively accurate and quick *in vitro* Lf determination. It soon became clear to us, however, that the " false " zones were so far removed from the " true " zone, in pH 11 extracts by factors of 8- and 32-fold, that in practice there was little danger of confusion. With a little experience and with a knowledge of the likely range of toxin concentration to be encountered the true toxin zone could be readily distinguished. In practice the false zones were never encountered after the preliminary work reported in Tables I and II because the toxin concentration in a given solution was never over-estimated by a factor as great as 8-fold, and consequently the antiserum concentration used in flocculation tests was never high enough to embrace the false zones. Zones III and IV (Table II) were also never again encountered because there was no virtue in extracting the toxin at pH 13.

A further guide to the true zone lies in the product, units antitoxin per ml. toxin-antitoxin mixture × flocculation time (min.). These values are given in brackets in Tables I and II. For the toxin zone it was always near 1000, whereas it was considerably higher or lower for the false zones.

The validity of the Lf was from time to time checked by a determination of the Lf: L+ ratio and this was never found to be below 0·6; generally it was between 0·65 and 0·75. In any event, the problem of the false zones became academic as soon as the non-toxic antigens were removed in the purification of the toxin.

LD_{50} determinations.

The LD_{50} is always predicted from the Lf, and serial 2-fold dilutions of toxin solution, equally spaced about the predicted value, are injected into the animals. With mice (20 g.)

0·5 ml. volumes of 7 dilutions are used and each dilution is injected intraperitoneally into 8 animals. With rabbits (*c.* 2·5 kg.) 0·2 ml. volumes of 5 dilutions are used and each dilution is injected into the ear veins of 4 animals.

The animals are observed for seven days, and those that survive this period are assumed to have survived the injection. The value of the LD_{50} is calculated by the method of Reed and Muench (1938).

L+ determinations.

The L+ is also predicted from the Lf and is determined at the "5 unit level." Seven mixtures, each containing 10 units antitoxin/ml. and amounts of toxin falling in a ratio of 1·25, are made up and 0·5 ml. volumes of each mixture are injected intraperitoneally into 8 mice. These animals are also observed for 7 days. The value of the L+ is also calculated by the method of Reed and Muench (1938).

Electrophoresis.

Zone electrophoresis on filter-paper (barbital buffer, pH 8·6, $\mu = 0{\cdot}1$) was carried out by different methods according to the objects in mind. For the determination of electrophoretic homogeneity the filter-paper was horizontally suspended in a chamber in a method generally similar to that of Plückthun and Götting (1951). After staining with bromphenol-blue the paper strips were scanned at 2·5 mm. intervals through a 3 mm. slit in a photo-electric device (van Heyningen, 1952, unpublished).

This method of electrophoresis gives good separation of components, and on scanning of the filter-paper strips neat diagrams can be obtained. These are useful, provided the limitations of the technique are understood. The most important of these is the fact that rate of migration of bands is not uniform owing to evaporation of water from the strip and consequent alterations in the field strength. For the determination of electrophoretic mobilities the method of Kunkel and Tiselius (1951) was used. In this technique the strip of filter-paper is clamped between sheets of glass. Evaporation and overheating are thus avoided and the rate of migration of protein spots on the paper is uniform. For incompletely explained reasons large spots or bands of protein become distorted as they migrate, and therefore lead to a broadening of the areas they occupy. For the determination of electrophoretic mobility, however, only small spots of protein solution need be placed on the paper, and these do not present difficulties.

Other Estimations.

Iron was determined in the usual way with α-α′-dipyridyl, after adsorption on calcium phosphate and nitrogen by the micro-Kjeldahl technique. Values of pH were determined electrometrically with a glass electrode. When the pH of a solution was adjusted to a value greater than 9·5, KOH was used in order to minimise deterioration of the electrode, and indeed very little deterioration was observed. High values of pH quoted in this paper, *e.g.*, pH 11, 13, are the readings obtained on the pH meter; they proved to be reproducible, but they may not represent the actual pH values. Protein content of toxin solutions was estimated by a spectrophotometric method recommended by Kalckar (1947). The optical densities of solutions at 260 mμ. and 280 mμ. were determined in a Beckmann Model DU photo-electric absorption spectrophotometer, and protein content was derived from the expression mg. protein/ml. $= 1{\cdot}45\ D_{280} - 0{\cdot}74\ D_{260}$. The dry weights of protein solutions were from time to time determined directly by evaporating 2 ml. samples on watch glasses on a water bath for 1 hr. and standing overnight in a P_2O_5 desiccator before weighing. These values always agreed within 10 per cent or less with the spectrophotometric determinations, but refractometric estimations on a solution of purified toxin showed that the dried specimens apparently retained about 25 per cent water (Baldwin, 1953). The protein concentration quoted in this paper for the purified toxin is the value obtained by the refractometric method, but other values are those determined spectrophotometrically.

EXPERIMENTAL.

Extraction of Toxin.

The neurotoxin of *Sh. shigae* is usually obtained in a soluble form by allowing the organisms to autolyse, but in our experience the yield obtained in this way

was very variable. We therefore resolved to use a method that was more readily controlled. Dubos and Geiger (1946) precipitated chilled cultures at pH 4·2, and extracted the toxin from the washed precipitate at pH 7·5 with dilute sodium carbonate or with salt solution buffered with phosphate. We found that more efficient extraction of toxin could be obtained at pH 11.

Fig. 1 shows that the optimum pH for extraction is 11. At higher pH no more toxin is extracted, but more non-toxic antigens appear, as is evident from Table II. Fig. 2 shows that the optimum time for extraction is 2-3 hr. The

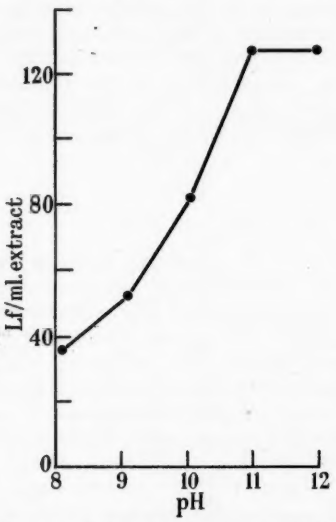

FIG. 1.—Effect of pH on extraction of the toxin.

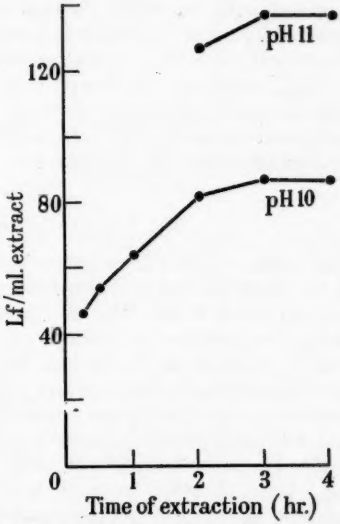

FIG. 2.—Effect of time of extraction on yield of the toxin.

yield of toxin is about 2 Lf/mg. organism, which is 50–120 per cent efficient as judged by a comparison of the LD_{50} content of the organisms and the extracts. However, when much greater volumes of extracting solutions are used 6–7 Lf may be extracted per mg. organism (*see* van Heyningen and Gladstone, 1953). The toxin does not appear to be adversely affected by the comparatively high pH of extraction, because the ratios of Lf to L+ and mouse LD_{50} (about 0·7 and 7 respectively) are no smaller than those in extracts obtained by autolysis or grinding at neutral pH (see below).

The routine method for extracting the toxin is as follows: the cultures are centrifuged down, washed with water and suspended in phosphate (potassium salts only) buffer pH 6 to a concentration of 50–60 mg. dry wt. organisms/ml. The organisms are killed by heating at 56–59° for 30 min. and the suspension is then adjusted to pH 11 with N KOH. After standing at room temperature for 3 hr. the suspensions are neutralised and spun down. The residue is re-suspended in half the original volume and re-extracted and the turbid greenish-yellow extracts are combined.

The killing of the organisms by heating at 56–59° for 30 min. is an essential stage in the extraction because no detectable toxin is extracted when this stage is omitted. We have confirmed an observation by Boivin, Delaunay and Sarciron (1940) that about 30 per cent of the toxin passes from the cells into the suspending medium on heat-killing. This process may be related to the extractability of the toxin, but neither of the phenomena is understood. Microscopic examination of smears of the heated and extracted organisms did not show any obvious evidence of destruction.

The toxin can also be extracted by grinding the cells. Packed washed cells were mixed with an equal volume of fine aluminium oxide powder (200 mesh) and ground for 3 min. by hand in a mortar. The residue of organisms and aluminium hydroxide was then extracted with phosphate buffer pH 8·6 and 60–130 per cent (LD_{50} yield) of the toxin was recovered. Heat-killing of the organisms was not necessary for this extraction. The ground organisms also did not appear to have been disintegrated. However, the alkaline extraction method is a more convenient technique for routine use.

Purification.

The crude turbid yellow-green solution obtained by extracting the organisms at pH 11, when diluted and clarified by high speed centrifugation, absorbs strongly with a maximum at 260 mμ. and presumably contains nucleic acid. The extract is rendered colourless by dialysis overnight against running tap water and then clarified by adding 0·1 vol. 0·2 M Na_2HPO_4, 0·05 vol. M $CaCl_2$, adjusting the pH to 7·6 and centrifuging the calcium phosphate precipitate after standing for 30 min. The resulting clear colourless supernatant no longer absorbs strongly at 260 mμ. but has a typical protein absorption spectrum with a maximum at 277 mμ. The yield of toxin is 80–90 per cent, with an activity of about 50 Lf/mg. protein. A typical electrophoretic analysis is shown in Curve 1, Fig. 3. At this stage the preparation is freeze-dried and material is accumulated for further purification.

Attempts at fractionating this complex mixture of proteins by precipitation with methanol at controlled temperature, pH and ionic strength, or by salting out with ammonium sulphate or sodium phosphate were not encouraging. However, it was noticed that the toxin was precipitated when a solution of the crude material was dialysed against distilled water, and this property was used in further purification. A 5 per cent solution of the freeze-dried powder in 1 per cent NaCl was dialysed against several changes of distilled water at 4°. After 10–20 days 30–90 per cent of the toxin precipitated out, with an activity of about 600 Lf/mg. protein. Zone electrophoretic analysis (Curve 2, Fig. 3) showed that the preparation was still complex. The precipitate was dissolved in 1/10 the original volume of 1 per cent NaCl, leaving an appreciable insoluble residue, and dialysed again against distilled water. A copious precipitate started forming within 30 min. and after overnight dialysis the yield of toxin in the precipitate was 60–90 per cent. After two further precipitations by dialysis, each with a 60–90 per cent yield, a preparation containing 2500–3000 Lf/mg. protein was obtained. Curve 3, Fig. 3, shows that a dominant component now appeared on electrophoretic analysis. This zone (from 5 to 9 cm. along the paper strips) was cut out and eluted with 1 per cent saline. The eluate contained 80 per cent of the toxin and no toxin could be recovered from the rest of the strip. The

barbiturate in the eluate interfered with the spectrophotometric estimation of the protein content, but the area occupied by the toxin zone corresponded to 70 per cent of the total area under the electrophoretic curve. From this it followed that electrophoretically homogeneous toxin should contain about 4000 Lf/mg.

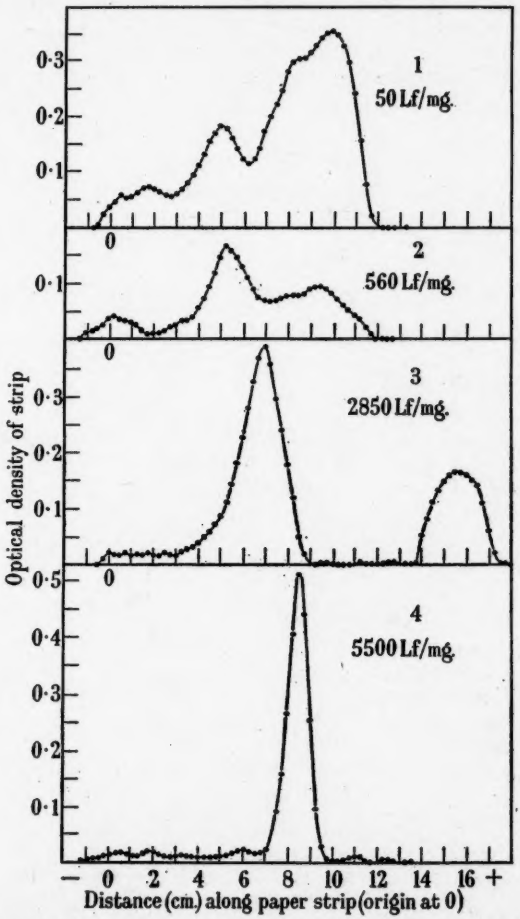

FIG. 3.—Electrophoresis (pH 8·6) of toxin preparations at different stages of purification.

protein, provided that all the proteins in the mixture adsorbed the same amount of dye.

Further precipitation by dialysis did not result in further purification, but the fast-moving component could be removed by adsorption on calcium phosphate precipitated in the manner described above. The yield at this stage was 60–90

per cent and the preparation contained 4150 Lf/mg. protein, when the protein content was determined spectrophotometrically, or 5500 Lf/mg. protein by the refractometric method. On zone electrophoresis at pH 5·6, 8·6 and 10·1 the toxin appeared to move as a single component (Fig. 4). These curves were obtained by eluting 1 cm. transverse sections of the paper strips and determining the protein content of the eluates. This technique, however, is not sensitive enough to detect very small amounts of protein.

The tail which can be seen in Curve 4, Fig. 3 was invisible to the naked eye on the paper strip and could be detected only with the photo-electric scanning

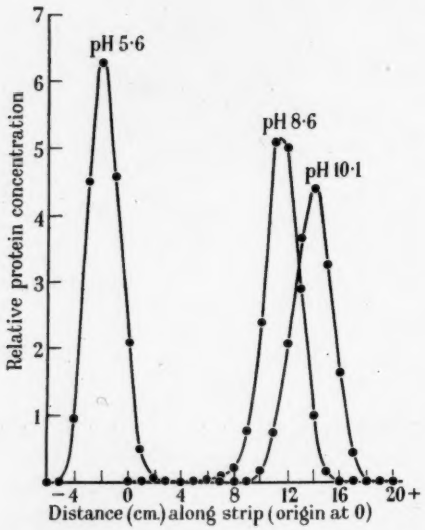

FIG. 4.—Electrophoresis of purified toxin at different pH values.

device. It may be due to retarding of the main component by adsorption on the paper, a common phenomenon in paper electrophoresis, or it may represent other components. The homogeneity of the preparation in sedimentation in the ultracentrifuge is discussed by Baldwin (1953). We were unable to crystallize the toxin.

The purification of the toxin requires at least 6 stages, *viz.*, extraction, clarification, 3 precipitations by dialysis and adsorption of impurities on calcium phosphate. At each of these stages a 90 per cent recovery of toxin was obtained at one time or another, but in any single purification from organisms to final product we never succeeded in getting six successive yields of this magnitude. On the average the stage yield was 70 per cent, and consequently the overall yield was about 12 per cent. The concentration of toxin in the organisms is about 1/1000; from 1000 l. of a culture grown on a de-ferrated medium and containing 2 mg. of organism/ml., it should be possible to recover about 100 mg. of purified toxin.

PROPERTIES.

Examination in the Ultracentrifuge.

The purified preparation was studied in the ultracentrifuge by Mr. R. L. Baldwin (1953). The sedimentation constant, diffusion coefficient, frictional coefficient and molecular weight as determined by him are given in Table V.

Nitrogen Content.

A Kjeldahl nitrogen estimation was made on a solution of the purified preparation, the protein content of which had been determined refractometrically (Baldwin, 1953). The preparation contained 15·7 per cent N.

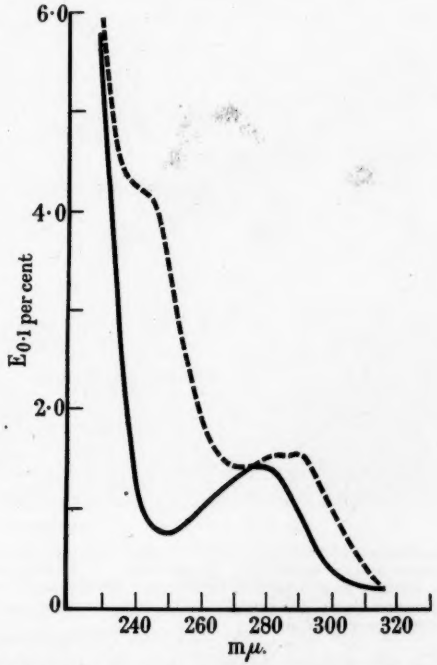

FIG. 5.—Absorption of ultra-violet light by purified toxin preparation. Continuous line, in saline pH 7; broken line, in 0·1 N NaOH.

Ultra-violet Absorption Spectra

The ultra-violet absorption spectra of the purified material dissolved in 1 per cent NaCl and in 0·1 N NaOH are shown in Fig 5. They are typical of proteins. From Holiday and Ogston's (1938) formulae, it was calculated that the tyrosine and tryptophan contents of the purified preparation are approximately 9·8 per cent and 2·5 per cent respectively.

Amino Acids.

Dr. G. G. F. Newton of this Department kindly carried out a qualitative chromatographic analysis on an acid hydrolysate of the preparation. A Pyrex glass tube containing 2 mg. with 1 ml. 6N HCl was sealed and heated at 110° for 18 hr. The HCl was then evaporated in a stream of air at 100°, and the residue taken up in water to give a solution of 300 μg. in 5 μl., of which 10 μl. were applied to Whatman No. 1 paper (18·5″ × 22·25″) and the chromatogram was first developed with butanol acetic acid (Woiwod, 1949) overnight. The

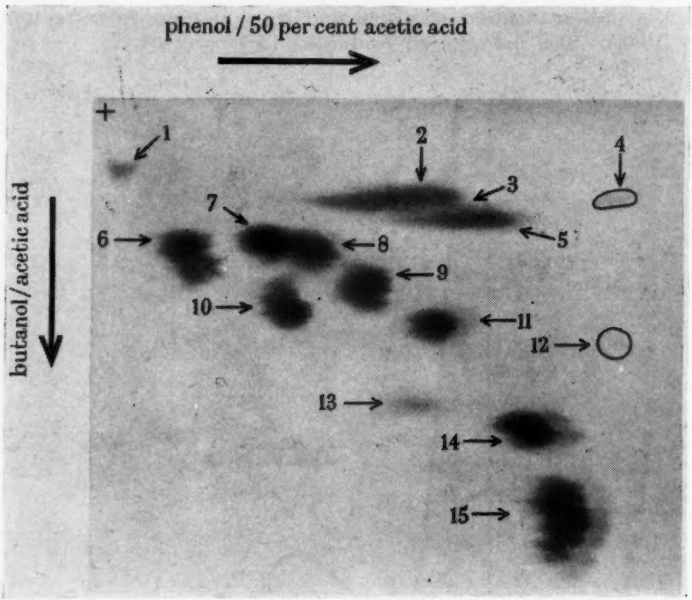

FIG. 6.—Paper chromatogram of acid hydrolysed purified toxin preparation. 1, cysteic acid (= cysteine); 2, lysine and/or ornithine; 3, histidine; 4, unidentified; 5, unidentified; 6, aspartic acid; 7, serine; 8, glycine; 9, threonine; 10, glutamic acid; 11, alanine; 12, proline and/or hydroxyproline; 13, tyrosine; 14, valine and/or methionine; 15, leucine and/or isoleucine and/or phenylalanine.

paper was then dried in a stream of air at 80° for 30 min. and developed in a second dimension with 80 per cent phenol in an atmosphere of 50 per cent acetic acid (Dent, 1948). The phenol was removed in a stream of air at 30–40° for 6–8 hr., the paper was sprayed with 0·2 per cent ninhydrin solution containing 0·2 per cent collidine (Woiwod, 1949), and the colour developed by heating at 105° for 5 min. The resulting chromatogram is shown in Fig. 6. In addition to the 15 or 16 amino acids seen in the chromatogram the ultra-violet absorption spectra suggest that the preparation contains about 2·5 per cent tryptophan which would be destroyed during the acid hydrolysis.

Solubility.

The toxin is insoluble in distilled water and soluble in aqueous salt solutions. This is evident from the method of purification described above. The solubility of the toxin in salt solution has a high temperature coefficient. If a 1 per cent solution of the crude toxin (after the first calcium phosphate clarification) is rapidly cooled to $-5°$ a precipitate is formed. If this precipitate is collected and re-suspended in 1 per cent NaCl a major portion of the toxin will dissolve, leaving an appreciable insoluble residue. The activity per unit dry weight of the resultant toxin solution may be increased by a factor of 10-fold, but the yield is poor.

Electrophoretic Mobility.

The electrophoretic mobility of the toxin was determined by the method of Kunkel and Tiselius (1951). Samples of the toxin and of human serum albumin were laid on a single strip of paper (Whatman No. 1) and allowed to move simultaneously in an electric field. The experiment was done at different pH values and corrections were made for the endosmotic effect by observing the movement of glucose spots. Since the mobility of albumin in free solution is known the corresponding values for the toxin could be calculated. These are shown in Table III. At pH 8·8 the toxin has about the same mobility as human serum β-globulin.

TABLE III.—*Electrophoretic Mobilities of Shiga Neurotoxin on Filter-paper and in Free Solution at Different pH Values.*

pH.	Buffer ($\mu = 0{\cdot}1$).	Electrophoretic mobility, $-u_3°$ (cm.^{2} sec.$^{-1}$. volt^{-1} × 10^5).	
		On Whatman No. 1 paper.	Calc. for free solution.
10	Barbital	3·30	3·76
8·8	,,	2·44	2·78
7·0	Phosphate	1·21	1·38
6·0	Phthalate	0·81	0·92
5·7	,,	0·58	0·66

Since the toxin was strongly adsorbed on the filter paper at pH values below 5·7 it was not possible to determine the iso-electric point by this method. The toxin appears to be least soluble at about pH 4.

Toxicity.

The purified preparation containing 5500 Lf/mg. was found to contain 750 LD_{50}/mg. per kg. mouse, 1,150,000 LD_{50}/mg. per kg. rabbit (see Table IV), and 4200 L+/mg. measured at the 5 unit level. Since the Lf : L+ and Lf : LD_{50} ratios of the purified material were not significantly different from those of crude extracts it was clear that no toxoiding had taken place during purification.

Like botulinus and tetanus neurotoxins Shiga neurotoxin is slow in its action. Rabbits and mice generally died on the 3rd and 4th day after injection of the toxin. Mice did not show any obvious signs before dying, but rabbits showed marked paresis. They became paralysed first in the forelimbs, then in the hind limbs, and finally became comatose. Several rabbits injected with sub-lethal doses of toxin showed almost complete paralysis on the 4th day after injection,

TABLE IV.—*Protocols for LD_{50} Determinations with Purified Shiga Neurotoxin* (5500 *Lf/mg.*, 4200 *L+/mg.*).

LD_{50} in mice (20 *g.*):

Dose (μg.)	0·25	0·125	0·0625	0·0313	0·0157	0·0078
Deaths.	8/8	7/8	8/8	6/8	0/8	0/8

LD_{50} (Reed and Muench, 1938) = 0·027 μg. per mouse
= 1·35 μg. per kg. mouse.

LD_{50} in rabbits (2·65 kg.):

Dose (μg.).	Deaths.	Remarks.
0·003	3/6	1 dead in 2 days, 2 dead in 3 days, 1 paralysed and recovered.
0·0015	2/4	2 dead in 3 days, 1 paralysed and recovered.
0·00075	0/4	1 paralysed and recovered.
0·00038	0/4	—

LD_{50} (Reed and Muench, 1938) = 0·0023 μg. per rabbit.
= 0·00087 μg. per kg. rabbit.

and would have died of thirst and hunger had they not been carefully nursed. They gradually recovered and after a further 8–10 days appeared to be quite normal.

Like botulinus and tetanus neurotoxins, but unlike most bacterial toxins, Shiga neurotoxin has no general histotoxic effect. A rabbit injected intracutaneously with nearly 400 LD_{50} showed no dermonecrosis and died 4 days after injection. Nor does it appear to be toxic by mouth; when 1000 intraperitoneal lethal doses were fed to mice no harmful results were observed. This has not yet been tested on rabbits.

Summary of Properties.

The properties of the purified preparation of Shiga neurotoxin are listed in Table V.

DISCUSSION.

The purified preparation of toxin appears to move essentially as a single zone on filter-paper electrophoresis, but thus far no protein which has been reported homogeneous in electrophoresis has in fact been shown to be so by the test of

TABLE V.—*Properties of Shiga Neurotoxin.*

Toxicity, per kg. mouse	750 LD_{50}/mg.
,, per kg. rabbit	1,150,000 LD_{50}/mg.
N content	15·7 per cent
Electrophoretic mobility, $-u_{2^0}$ (barbital buffer, pH 8·8, $\mu = 0·1$)	$2·78 \times 10^{-5}$ cm.^{2} sec.$^{-1}$, volt^{-1}
Average sedimentation coefficient, Sm_{20}, w.*	4·80 $\pm$ 0·05 S
Diffusion coefficient, D_{20}, w.*	$5·7 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ $\pm$ 5 per cent
Frictional coefficient, f/fo.*	1·26
Average molecular weight.*	82,000

* From Baldwin (1953).

boundary spreading (Alberty, Anderson and Williams, 1948). Although the material has been carefully separated and gives a single fairly symmetrical boundary in the ultracentrifuge (like the other purified toxins in Table VI) it has not been found by Baldwin (1953) to be homogeneous with respect to its sedimentation coefficient.

The physical heterogeneity of our preparation may mean that a homogeneous active material is still contaminated with other inactive material, or that a degree of heterogeneity has been produced in the course of its separation; it may be on the contrary, that the active material was physically heterogeneous in its original state. There is no good reason for supposing that the toxin must be homogeneous.

The toxicity of the purified material was surprisingly high. Table VI shows that it is considerably more toxic than diphtheria toxin and as toxic as the two

TABLE VI.—*Comparison of Toxicity of Highly Purified Bacterial Toxins.*

Toxin	LD_{50}/mg. per kg. animal.	Animal.
Diphtheria	3·5	Mouse
	3,500	Guinea-pig
Botulinus Type A	620,000	Mouse
	1,200,000	Guinea-pig
Tetanus	200,000	Mouse
	1,200,000	Guinea-pig
Shiga neurotoxin	750	Mouse
	1,150,000	Rabbit

(For references to diphtheria, botulinus and tetanus toxins, see Pappenheimer (1948).)

most toxic substances hitherto known, botulinus and tetanus toxins. Dubos and Geiger (1946) reported that per unit weight rabbits were 75–150 times more susceptible to the toxin than mice, but in our experience this ratio was greater than 1000. The concentration of the toxin in the organism is only about 1/1000, and this accounts for the fact that cultures of *Sh. shigae* are not considered particularly toxic when compared with other toxin-producing organisms.

There can be little doubt that the highly neurotoxic protein of *Sh. shigae* is a different substance from the considerably less toxic polymolecular complex of the O antigen of the smooth strains. The view may still be held that the neurotoxin is the protein component of the O antigen, but in that event its toxicity would have to be considerably reduced by the attachment of the phospholipid and polysaccharide components. That this is not altogether impossible is perhaps suggested by the work of Boivin, Delaunay and Sarciron (1940), who found that the O antigen content of autolysates of smooth strains remained constant on standing while the neurotoxin content increased up to 20-fold. This is not inconsistent with the view that a negligible amount of O antigen may have decomposed to liberate a very small amount of extremely potent neurotoxin.

SUMMARY.

The neurotoxin has been extracted from a rough strain of *Sh. shigae* and obtained in an electrophoretically homogeneous state. The purified preparation contains 5500 Lf/mg., 4200 L+/mg., 750 LD_{50}/mg. per kg. mouse and 1,150,000 LD_{50}/mg. per kg. rabbit. Its physical and chemical properties have been investigated.

We are greatly indebted to Mr. J. C. Macsween of the Microbiological Research Department (Ministry of Supply), Porton, Wiltshire, for producing the large

quantities of *Sh. shigae* culture necessary for this work, and to Dr. D. W. Henderson, the Director, for his willing co-operation in allowing the facilities of the Department to be used for this purpose. We have to thank Professor C. L. Oakley and the Wellcome Research Laboratories, Beckenham for a generous supply of Anti-Dysentery Serum, Mr. R. L. Baldwin for investigating the behaviour of the toxin in the ultracentrifuge, and Dr. G. G. F. Newton for carrying out a chromatographic analysis of the toxin. Our gratitude is also due to Miss A. M. Holmes who was actively associated with the early part of this work, and to Miss W. Crawford who has given able technical assistance throughout.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

2. Examination of the Toxin in the Oil-turbine Ultracentrifuge.

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Received for publication December 17, 1952.

A sample of Shiga neurotoxin, prepared by van Heyningen and Gladstone (1953), was furnished for physico-chemical examination. The difficulty of obtaining more than a few mg. of this toxin made it desirable to obtain as much information as possible from few measurements. It has proved possible to obtain the concentration, the weight-average sedimentation coefficient, the distribution of sedimentation coefficient, and a mean diffusion coefficient, all from two ultracentrifuge experiments made on a single (0·7 ml.) sample of solution. The contributions of diffusion and of heterogeneity to the spread of a boundary in the ultracentrifuge can be sorted out, and both the diffusion coefficient and the distribution of sedimentation coefficient obtained, by making use of the different way in which these two vary with time.

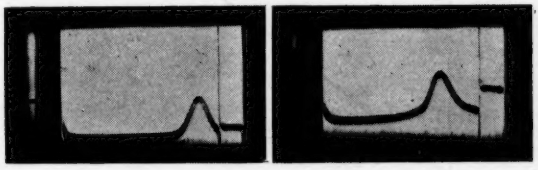

Fig. 1.—Schlieren diagrams of the sedimentation of Shiga neurotoxin (0·5 g./100 ml.). The fine vertical line to the right of the sedimenting peak represents the meniscus between toxin solution and liquid paraffin (*a*) after sedimentation for 55 min. at 650 rev./sec. (*b*) after sedimentation for 58 min. at 900 rev./sec. Much of the skewness is due to buffer sedimentation.

The diffusion coefficient is found by plotting an "apparent diffusion coefficient," D^* (Williams, Baldwin, Saunders and Squire, 1952), against time and extrapolating to zero time. For a homogeneous substance this plot will be a horizontal line. When the boundary includes substances of differing sedimentation coefficient, D^* will increase with time in a nearly linear fashion; the slope of this plot is a measure of the degree of heterogeneity. The distribution of sedimentation coefficient is found by a method comparable to that of finding the diffusion coefficient (Williams *et al.*, 1952; Gosting, 1952): an "apparent distribution of sedimentation coefficient" is obtained from each boundary record and these are again extrapolated, but now to infinite time.

EXPERIMENTAL.

Toxin, 4·5 mg., was dissolved in 0·65 ml. of buffer (0·2 M NaCl, 0·0077 M Na_2HPO_4, 0·0023 M KH_2PO_4; pH measured by glass electrode = 7·25 at 20°).

This volume was sufficient nearly to fill a 12 mm. ultracentrifuge cell: the solution was covered with a layer of liquid paraffin. Two runs were performed in the Svedberg oil-turbine centrifuge, the first at 650 rev./sec.; after this run the cell contents were mixed by stirring, and a second run was performed at 900 rev./sec. The procedure was that of Cecil and Ogston (1948), except that the edge-schlieren optical system was replaced by a bar-schlieren system (Fig. 1). This was adjusted so as to give a narrow boundary trace, whose centre could be measured with high precision. Values of X and dn/dx were measured with a two-way travelling microscope. Base lines were separately determined, and base line correction was made to the values of dn/dx.

RESULTS AND DISCUSSION.

1. The area under the boundary curves was obtained by summation of dn/dx; this gave a refractive increment for the original solution of $0{\cdot}97 \times 10^{-3}$ ($\pm$ 1 per cent). Assuming a value for the specific refractive increment of $1{\cdot}85 \times 10^{-3}$ (this is unlikely to be wrong by more than 3 per cent), the concentration of the

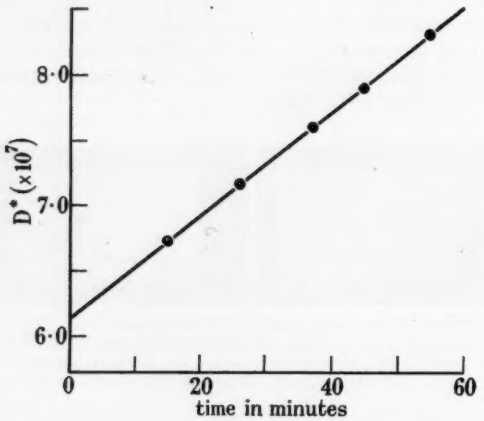

FIG. 2.—Extrapolation of the "apparent diffusion coefficients" from the centrifuge run at 650 rev./sec. to zero time. The values shown refer to the viscosity of the buffer at the temperature of the centrifuge run.

solution is 0·52 g./100 ml., compared with an expected value (from the wt. of toxin used) of 0·69 g./100 ml. It is possible, however, that some part of the toxin might sediment too fast or too slowly to appear in the sedimentation diagram.

2. For obtaining the diffusion coefficient the centrifuge run at 650 rev./sec. was used, since diffusion is relatively more pronounced at lower speed. The extrapolation of D^* to zero time is shown in Fig. 2. D^* was calculated from the boundary curves by a method to be described in detail elsewhere; in addition to the factors discussed by Williams *et al.* (1952), explicit allowance was made for the dependence of sedimentation coefficient upon concentration and for the possibility of convection while the centrifuge is coming up to speed. D^* values were obtained by the "height-area" method, since the boundary did not depart

significantly from Gaussian shape during the time these measurements were taken. The value obtained for the diffusion coefficient, $D_{20,\ w}$, is $5{\cdot}7 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ with an estimated uncertainty of $\pm$ 5 per cent.

3. For obtaining the distribution of sedimentation coefficient, by extrapolation to infinite time, the centrifuge run at 900 rev./sec. was used. The resulting distribution is shown in Fig. 3. The weight-average value of the sedimentation coefficient, $S_{m20,\ w}$, is $4{\cdot}80 \pm 0{\cdot}05$ Svedberg units, and the standard deviation of its distribution 0·83 S.

4. Using the mean values of S and D, and assuming the partial specific volume to be 0·75 ml. per gm., the value for molecular weight M is 82,000. It is not

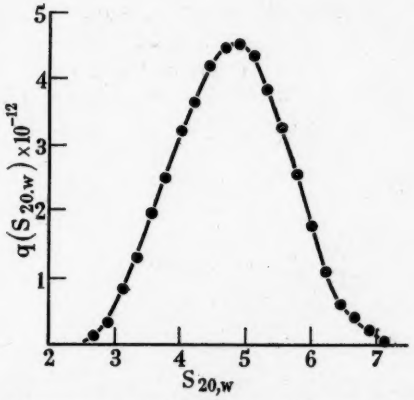

FIG. 3.—Distribution of sedimentation coefficients, obtained at a concentration of 0·5 g./100 ml.

possible to say from these data whether the toxin possesses a distribution of molecular weight comparable to the distribution of sedimentation coefficient, since the latter could be caused by variation in shape (*i.e.*, frictional coefficient) as well as molecular weight; the mean value of molecular weight given here is not a simply defined average. Additional uncertainty in the molecular weight arises from the fact that the measurements were made at finite concentration. Since the concentration was only 0·5 g./100 ml. and the value of f/f_0 only 1·26, it is unlikely that the last source of error is important.

One general comment is required. The breadth of the distribution of sedimentation coefficient found here should be a warning against judging, from the appearance of a single, fairly symmetrical boundary in the ultracentrifuge, that the substance is homogeneous in sedimentation.

SUMMARY.

The concentration, the mean sedimentation coefficient and its distribution and a mean diffusion coefficient of a sample of Shiga neurotoxin have been determined from two experiments in a Svedberg ultracentrifuge. Only 4·5 mg. of material was required for these measurements.

I would like to acknowledge Dr. A. G. Ogston's helpful advice and suggestions throughout the course of this work, which is part of a thesis to be presented for the degree of Doctor of Philosophy at Oxford University.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

3. The Effect of Iron on Production of the Toxin.

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Received for publication December 17, 1952.

Dubos and Geiger (1946) showed that the production of neurotoxin by rough and smooth strains of *Shigella shigae* was inhibited when iron was present in the culture medium. This discovery accounted for earlier observations that more toxic organisms are produced when they are grown in broth at moderately high pH values. In the normal preparation of broth alkaline conditions would lead to precipitation of calcium phosphate and adsorption of iron. In de-ferrated media toxin can be produced at neutral pH. The production of diphtheria toxin (Pappenheimer and Johnson, 1936), tetanus toxin by some strains of *Clostridium tetani* (Mueller and Miller, 1945), *Cl. welchii* α-toxin (Pappenheimer and Shaskan, 1944) and staphylococcus α-toxin (Proom, 1950, personal communication) is also inhibited by iron. The production of diphtheria toxin per unit volume of culture filtrate is at its maximum when the iron content of the medium is about 0·1 μg. Fe/ml; below this value toxin production is decreased because of diminished growth of the organism. Pappenheimer (1947) showed that for every 4 atoms of iron added to the medium above this value 4 less molecules of extra-cellular porphyrin and 1 less molecule of toxin are produced. The iron is taken up by the organism, which at the same time acquires an increased content of cytochrome *b*, a respiratory pigment which is associated with the cellular oxidation of succinic acid. Pappenheimer and Hendee (1947) showed that the succinoxidase activity of the organism is inversely proportional to its toxin production, and their data have been re-calculated and plotted in Fig. 1. This figure shows that when toxin production has fallen to about half its maximum value succinoxidase activity rises to nearly half its maximum value. Pappenheimer suggested that diphtheria toxin may be the protein moiety of the bacterial cytochrome *b*. When the organism is grown in an iron-deficient medium it continues to synthesise the protein and porphyrin components, but since these cannot be incorporated in the respiratory pigment without iron they are excreted as waste products. The toxin is thought to exert its effect by competitively inhibiting the synthesis of cytochrome *b* in the tissues of the host. This suggestion has recently been supported by the experiments of Pappenheimer and Williams (1952).

It occurred to us that this hypothesis, or a modification of it, might conceivably also apply to the production of neurotoxin by *Sh. shigae*. Accordingly we have investigated the quantitative relationships between toxin production and iron content of the medium, and we have made a preliminary study of the respiration of succinic acid and other substrates by toxic and non-toxic organisms.

METHODS.

Cultures.

The rough strain of *Sh. shigae* and the CCY medium (Gladstone and Fildes, 1940) mentioned in an earlier paper (van Heyningen and Gladstone, 1953*a*) were used. The culture vessels were tubes shaped like an inverted T. This is a modification of an excellent device originally designed by Dr. Jacques Monod of the Pasteur Institute, to whom we are indebted (*see* Monod, Cohen-Bazire and Cohn, 1951). The horizontal limb of the T is a tube of 2·25 cm. external diameter and 16 cm. long, one end being rounded and the other invaginated by means of a sealed-in tube of 1·2 cm. external diameter and 4 cm. long. The invagination is used in connection with growth measurements described below. The vertical limb of the T is a tube of 1·75 cm. external diameter, 9·5 cm. long, and open at the end. The tube is charged with 5 to 12 ml. of medium, plugged in the vertical limb, and autoclaved and inoculated. It is then clamped on a rocking device with the centre of rotation 1 cm. below the plugged end, and continuously rocked in the plane of the tube through an angle of 35° at the

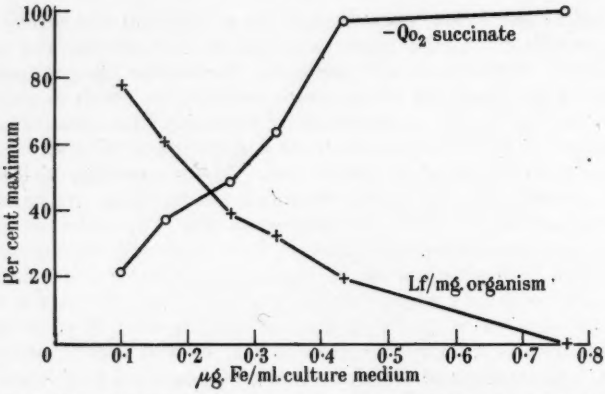

FIG. 1.—Effect of iron in culture medium on toxin production and rate of succinate oxidation by *Corynebacterium diphtheriae*. (Adapted from Pappenheimer and Hendee, 1947.)

rate of 56 oscillations/min. During rocking the tubes are immersed in a water bath at 37° to such a depth that about 2 cm. of the vertical limb protrude above water level. The rocking ensures complete aeration of the medium and even suspension of the culture. The rocking device carries 36 tubes and each culture is run overnight in duplicate.

Estimation of Growth.

For estimation of bacterial growth the tubes are wiped dry and the horizontal limb tilted to vertical so that the culture runs either into the rounded or the invaginated end. The end containing the culture is placed in a suitably adapted photo-electric absorptiometer, the invaginated end, in which the effective optical path is reduced, being used for heavier suspensions. Absorptiometer readings are then referred to curves calibrated, for either end, to yield figures expressing bacterial growth as mg. dry wt. organism/ml. Suitable corrections are made for the optical density of uninoculated media.

Toxin Assay.

The LD_{50} of washed suspensions of organisms was determined in mice according to the method already mentioned (van Heyningen and Gladstone, 1953*a*). The Lf content of small volumes of extracts of organisms was determined by the semi-micro flocculation assay of van Heyningen and Gladstone (1953*b*).

Other Estimations.

Iron was determined in the usual way with α-α′-dipyridyl after adsorption on calcium phosphate, and values of pH electrometrically with a glass electrode. Oxygen uptake ($-Qo_2$) of bacterial suspensions was measured in the usual way in Warburg manometers. Absorption spectra of bacterial suspensions were observed in a hand spectroscope.

EXPERIMENTAL.

De-ferration of Medium.

The CCY medium of Gladstone and Fildes (1940) is composed of the following constituents: 175 ml. acid hydrolysate of casein (A), 75 ml. tryptic digest of casein (B), 500 ml. yeast extract (C), 50 ml. 70 per cent (w/v) sodium lactate, 50 g. sodium glycerophosphate, made up to 5 l. with distilled water. The iron content of the medium varies from 3·5 to 5 μg.Fe/ml., this being contributed by the three main components roughly in the following proportions: A, 0·5–0·7; B, 0·15–0·3; C, 2–4 μg.Fe/ml. The medium was de-ferrated by precipitating calcium phosphate in the mixed concentrated components before dilution. To the amounts of components listed above were added 10 g. $Na_2HPO_4.12\ H_2O$, and after the pH was adjusted to 8·6, 25 ml. of M $CaCl_2$ was added with stirring. The mixture was then brought to boil, and the resultant precipitate filtered off and the precipitation repeated until the iron content of the diluted medium was reduced to about 0·05 μg.Fe/ml. The figures in Table I are typical. In order to reduce

TABLE I.—*Iron Content of CCY Medium after Successive De-ferrations by Means of Calcium Phosphate Precipitation.*

	μg.Fe/ml. medium.
Before de-ferration	4·63
After 1st de-ferration	0·31
,, 2nd ,,	0·09
,, 3rd ,,	0·05

the iron content still further the diluted medium was treated with Al_2O_3 according to the method of Donald, Passey and Swaby (1952). This resulted in a final value of approximately 0·025 μg.Fe/ml., but this figure must be accepted with some reserve since the determination of such small concentrations of iron is difficult.

Effect of Iron and Magnesium on Growth.

De-ferration also reduced the Mg-content of the medium to values which would not support growth of the organism and therefore it was necessary to add 20 μg.Mg per ml. of medium after de-ferration. Fig. 2 shows the effect of Mg and Fe on growth in de-ferrated medium in which, respectively, the Fe and Mg contents had been brought to the maximal level for growth. Filtrates of cultures grown in media containing up to 1 μg.Fe/ml. contain no detectable iron, and presumably the iron is taken up by the organisms. Above this level increasing amounts of iron are found in the culture filtrates. Fig. 3 shows the iron content of the organisms with increasing iron in the medium.

Effect of Iron on Toxin Production.

Fig. 4 shows, in confirmation of Dubos and Geiger (1946), that the toxicity of the organisms decreases as their iron content increases. This curve, however, provides little useful information because of the uncertainty of the LD_{50} determination. Therefore the organisms were extracted at pH 11 (van Heyningen and

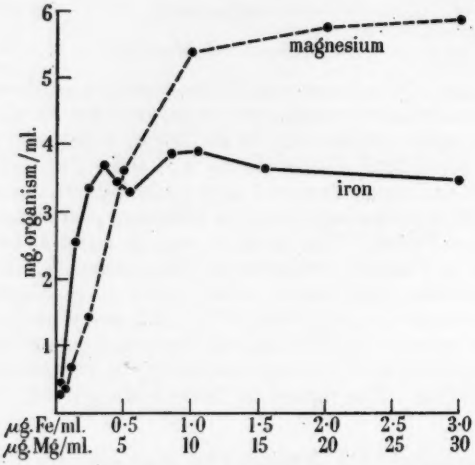

FIG. 2.—Effect of iron and magnesium on growth of *Sh. shigae* in media in which the magnesium and iron concentrations are 20 μg./ml. and 2 μg./ml. respectively.

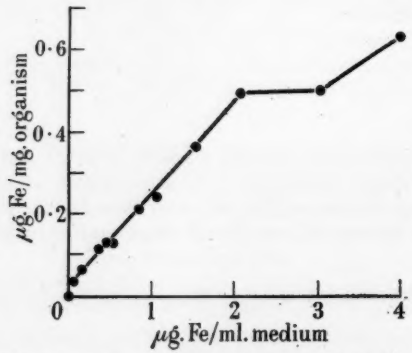

FIG. 3.—Relation between the iron content of the medium and the iron content of the bacterium in *Sh. shigae* cultures.

Gladstone, 1953*a*), and a means was devised of determining the Lf content of the small volumes of extract that were available (van Heyningen and Gladstone, 1953*b*). The experiment was done in the following way: duplicate culture tubes were each charged with 11 ml. of medium of increasing iron content, and after overnight growth of the organisms the contents of each pair of duplicates were

pooled. Volumes of 10 ml. of cultures at each iron level were centrifuged, and the organisms were washed and used for metabolic experiments described below. Other 5–10 ml. samples of each culture were adjusted to pH 6 and heated at 56–59° for 30 min. to kill the organisms. The suspensions of organisms were then adjusted to pH 11 with N KOH and neutralised after standing 2 hr. at room temperature. After centrifuging down the organisms the supernatant extracts were dialysed overnight against running tapwater and freeze-dried. The dried residues were dissolved in 0·2–1 ml. saline according to the expected Lf content of the extracts. It was generally necessary to centrifuge off a residue of insoluble material. The Lf contents of the extracts were then determined by means of the semi-micro flocculation assay, and the Lf content per mg. dry wt. of organisms calculated. The yields of toxin obtained in these extracts were always greater than those obtained in the large scale extractions (van Heyningen and Gladstone, 1953*a*) presumably because the ratio of the volume of extracting solution to weight of organisms was 30–40 times greater.

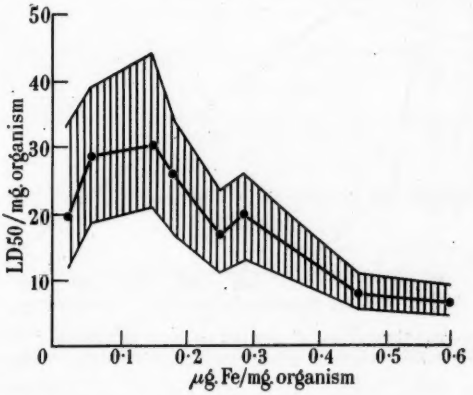

FIG. 4.—Relation between the iron content and the toxicity to mice of *Sh. shigae*. The hatched area represents the probable statistical limits of error in the LD_{50} determinations.

Table II shows typical figures relating Lf content of organisms to their iron content. From these figures, together with the values of 0·182 μg. for the weight

TABLE II.—*Effect of Iron on Toxin Production by* Sh. shigae.

μg. Fe/mg. organism.	Lf/mg. organism.
0·063	9·00
0·158	7·25
0·238	4·95
0·340	2·60
0·425	0·53

of 1 Lf of toxin (van Heyningen and Gladstone, 1953*a*) and 82,000 for the mean molecular weight of the toxin (Baldwin, 1953) it was possible to calculate how many

atoms of iron are required to inhibit the production of one molecule of toxin. In Fig. 5, Curve A, the data from Table II were re-calculated and plotted so as to show this relationship. This shows that within the range of iron concentrations starting at about 0·1 μg.Fe/mg. organism, or about 0·05 μg.Fe/ml. medium, 330 atoms of iron inhibited the production of one molecule of toxin. Curve B, Fig. 5, shows the data obtained when the experiment was repeated with a different range of iron concentration. Over the range of iron concentrations corresponding to that of Curve A the value of 330 atoms of iron per molecule of toxin is confirmed, but at lower iron concentrations only about 40 atoms of iron are required to inhibit the production of one molecule of toxin. It is possible that at still lower iron concentrations, below 0·04μg.Fe/mg. organism or about 0·025 μg.Fe/ml. medium, still less atoms of iron would be required to inhibit the production of a molecule

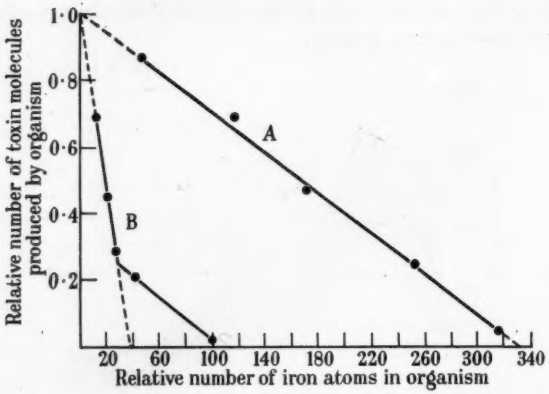

FIG. 5.—Data from two experiments (A, B) plotted to show the number of iron atoms required to inhibit the production of one molecule of neurotoxin by *Sh. shigae*.

of toxin, but this point would be difficult to test because of the difficulty of estimating such low values of iron and because growth of the organisms at these values is very small.

Effect of Iron on the Metabolism of the Organism.

We determined the rate of oxidation of a number of substrates (at 0·016 M concentration) by washed suspensions of organisms containing various amounts of iron, and in the case of lactate and succinate correlated these with the toxin content of the organisms. Fig. 6 shows that the rate of succinate oxidation increases with increasing iron content of the organisms and with their ability to grow, and is more or less inversely proportional to their toxin content. On the other hand, the rate at which the organism oxidizes lactate diminishes, if anything, with increasing iron content. The small initial rise in the rate of lactate oxidation, followed by a fall, is not fortuitous, since it was observed on each of the several occasions on which these experiments were done.

The rate of oxidation of isocitrate, α-ketoglutarate, malate and fumarate depended on the iron content in a similar fashion to that of succinate, but the

results with isocitrate and α-ketoglutarate must be accepted with some reserve, since the rate of oxidation by organisms of high iron content was low (see Table III). All these substrates are components of the tricarboxylic acid cycle. The

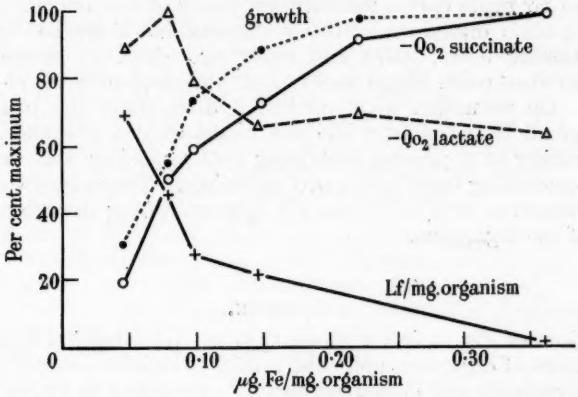

FIG. 6.—Effect of iron content of *Sh. shigae* on growth, toxin production and rates of succinate and lactate oxidation (cf. Fig. 1).

TABLE III.—*Effect of Iron Content of* Sh. shigae *on its Ability to Oxidize Various Substrates.*

	$-Qo_2$.		
μg.Fe/mg. organism.	0·048.	0·070.	0·690.
Lactate	185	357	240
Lactate + BCB*	330	350	240
Glucose	102	151	128
Pyruvate	93	183	145
Pyruvate + BCB*	0	0	0
Acetate	21	72	52
Isocitrate	0	—	13
α-Ketoglutarate	0	—	34
Malate	0	—	34
Fumarate	16	—	140
Succinate	11	45	140
Succinate + BCB*	0	0	0

* BCB = 0·08 per cent brilliant cresyl blue.

oxidation of substrates outside the cycle, *i.e.*, lactate, glucose and pyruvate, was more or less unaffected by the iron content of the organisms, except that they were all oxidized at a higher rate by organisms of intermediate iron content than by organisms containing smaller or greater concentrations of iron. Acetate occupied an intermediate position between these two groups of substrates. Table III also shows that in the presence of the oxygen-carrying dye, brilliant cresyl blue (Weil-Malherbe, 1937), the rate of oxidation of lactate by organisms of low iron content

was somewhat increased, whereas organisms of intermediate and high iron content were unaffected. On the other hand the oxidation of pyruvate and succinate was completely inhibited, irrespective of the iron content of the organisms. The significance of these findings is not clear.

We have so far made only a rudimentary study of the cytochrome content of toxic and non-toxic organisms. Heavy suspensions of washed organisms (26 mg./ml.) containing 0·048, 0·070 and 0·690 μg.Fe/mg. all showed apparently equally intense absorption bands at 630 mμ. and double bands at 562–564 and 566–568 mμ. On reduction with alkaline hydrosulphite the band at 630mμ. disappeared in all three cases, while the bands at 562–564 and 566–568 mμ. intensified strongly in organisms containing 0·69 μg.Fe/mg. and were unaffected in organisms containing 0·048 and 0·070 μg.Fe/mg. The reducible pigment may have some connection with toxin, since it appears during the greatest change in the toxicity of the organisms.

DISCUSSION.

A comparison of Fig. 1 and 6 shows that the relationships between the iron and toxin content of organisms and their ability to oxidize succinic acid are much the same in diphtheria and dysentery bacilli. According to Fujita and Kodama (1934) both organisms are rich in cytochrome *b*. But here the similarity ends. The diphtheria bacillus oxidizes glucose, maltose, lactate, fumarate, malate, glutamate and α-ketoglutarate at increasing rates as its iron content increases (Pappenheimer and Hendee, 1947), whereas *Sh. shigae* distinguishes markedly between substrates which fall within or outside the tricarboxylic acid cycle. The diphtheria bacillus produces porphyrin when it produces toxin ; the dysentery bacillus apparently does not produce porphyrin. The production of one molecule of diphtheria toxin is inhibited by 4 atoms of iron ; according to the concentration of iron in the organism from 40 to 330 atoms of iron are needed to inhibit the production of one molecule of Shiga neurotoxin, but it is possible that less iron may be required at lower concentrations.

We are not yet able to offer any suggestions as to the mechanism by which Shiga neurotoxin is produced, and it is clear that this interesting problem needs further investigation.

SUMMARY.

Up to a concentration of 1·5–2 μg.Fe/ml. culture medium the growth of *Shigella shigae*, its uptake of iron, and its ability to oxidize succinic acid and other substrate of the tricarboxylic acid cycle are directly dependent on the iron content of the medium.

The rates of oxidation of glucose, lactate and pyruvate do not increase with increasing iron content of the organism.

The toxin content of *Sh. shigae* decreases with increasing iron content. Over the range 0·1 to 0·4 μg.Fe/mg. organism 330 atoms of iron inhibit the production of one molecule of toxin ; over the range of 0·04–0·1 μg.Fe/mg. organism 40 atoms of iron are needed, and below this range it is possible that less iron may be needed.

The ability to oxidize succinic acid and the toxin content of the organism are inversely proportional.

We are grateful to Dr. J. M. Hammersley for a statistical evaluation of the data plotted in Fig. 4, and to Miss Winifred Crawford for competent technical assistance.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

4. A Semi-micro Method for the Flocculation Assay of the Toxin.

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From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication December 17, 1952.

In the Ramon flocculation assay of toxins (*e.g.*, *Shigella shigae* neurotoxin, diphtheria toxin, *Clostridium welchii* α-toxin) it is usual to employ 5 to 10 tubes, each containing 0·5–2 ml. of a fairly concentrated (10–100 Lf/ml.) toxin solution. In our investigation of the effect of iron on the production of neurotoxin by *Sh. shigae* (van Heyningen and Gladstone, 1953) it was necessary to extract the toxin from amounts of organism as small as 2 mg. dry weight, so that frequently not more than 0·2 ml. of toxic extract of appropriate strength was available. Consequently a semi-micro method for the flocculation assay was developed.

Ten 20μl. drops of the toxin solution are measured out with a constriction micropipette (Linderstrøm-Lang and Holter, 1940) and placed in the depressions of a greased spot test tile. To each drop amounts of antitoxin falling by 20 per cent are added with an " Agla " microsyringe, the largest volume of antitoxin solution being 4 μl. While the antitoxin solution is being expelled from the syringe the tip of the needle is held under the surface of the drop of toxin solution. The contents of each drop are mixed by drawing them up and down a capillary tube and finally discharging them again on the tile. A drop of dye solution and a drop of paraffin oil are also placed on the tile.

Samples, 2–3 μl. in volume, of each of the 10 toxin-antitoxin mixtures are now transferred to a single capillary tube, each pair of samples being separated by a 1–2 μl. volume of paraffin oil. This is done as follows: the steel needle of an " Agla " microsyringe is replaced by the butt end cut off from the glass delivery tip supplied with the syringe and the syringe is filled with paraffin oil. An 18 cm. length of capillary tube made by pulling Pyrex tubing of 1·4 cm. external diameter, 1·1 cm. internal diameter, to 1 mm. external diameter is inserted about 5 mm. into the butt on the microsyringe and held in position with a " Plasticine " seal. Paraffin oil is then expelled down half the length of the capillary tube, and successive samples of paraffin oil and toxin-antitoxin mixture, starting with the mixture containing the least amount of antitoxin, are drawn into the tube. The length of capillary occupied by each of the toxin-antitoxin mixtures is 2–3 mm. and by the oil 1–2 mm. The last sample of toxin-antitoxin is followed successively by oil, marker solution of dye, oil and air. The dye marker is placed in the tube to identify the end containing the mixture with the largest amount of antitoxin, and to distinguish different tubes from each other. More than one marker

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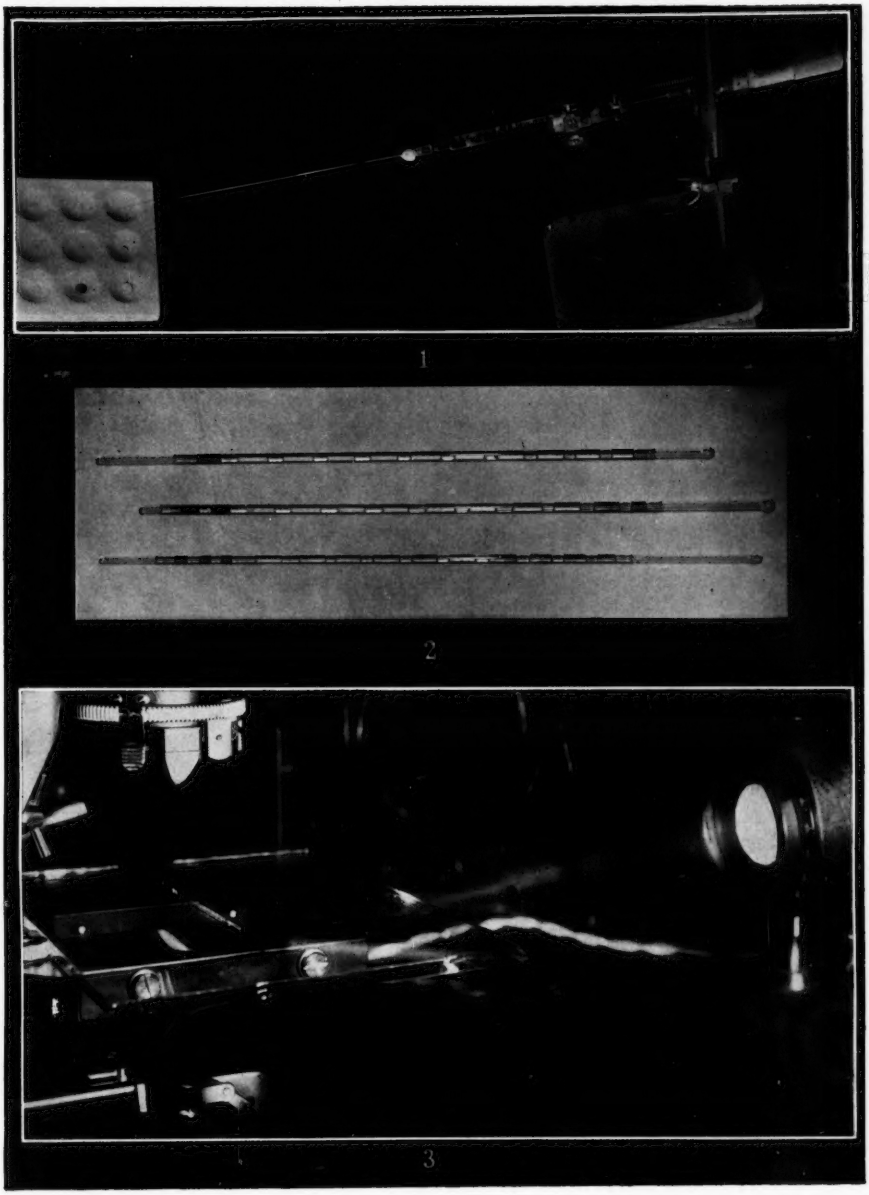
DESCRIPTION OF PLATE

Fig. 1.—Arrangement for drawing toxin-antitoxin samples into capillary tube.

Fig. 2.—Loaded capillary tubes, with dye markers on the left. (Approximately full scale).

Fig 3.—Arrangement for viewing capillary tubes.



van Heyningen and Gladstone.

may be used and the colour of the dye may be varied. The oil spacers are placed between the toxin-antitoxin mixtures to prevent them from breaking up or running together. The general arrangement of the apparatus is shown in Fig. 1. It will be seen that the plunger of the microsyringe is spring-loaded so that it follows the movement of the micrometer. The loaded tube is broken in the middle, about 1 cm. away from the first oil drop, and both ends are sealed in a micro-flame. Fig. 2 shows three loaded tubes, distinguished from one another by the number of dye markers they carry. It is possible to fill several replicate capillary tubes from one set of toxin-antitoxin mixtures.

The capillary is now placed in a water-bath at 50° and withdrawn from time to time for observation under a binocular stereoscopic plate microscope. The tube is placed on a sheet of plate glass, which rests on the stage of the microscope and can be moved from side to side by hand. Illumination is supplied by an almost horizontal beam of light focused on the edge of the sheet of plate glass. The arrangement of the microscope and its light source is shown in Fig. 3. This arrangement results in the tube being brightly illuminated against an almost black background. If the light beam is focused directly on the tube there is too much dazzle. In our experience a 2·5 × objective with 7 × eye-pieces proved to be the most satisfactory. According to the strength of the toxin solution flocculation takes place in 3–45 min., and the order of flocculation in the different mixtures is easily seen provided the spacing of antitoxin is 20 per cent. At this spacing nearly an 8-fold range can be covered with 10 mixtures.

It had been our intention at first to use finer capillaries and to observe them with a greater magnification on a dark field obtained with a dark-ground condenser in the expectation that unflocculated mixtures would appear black and flocculated mixtures grey, but this was not possible for two reasons: in the first place, it was found that toxin-antitoxin mixtures which flocculated within a few minutes in ordinary small test tubes would not flocculate, even after 24 hours' incubation, in fine capillaries. This was possibly due to there being no convection currents in the samples in the fine capillaries to hasten the aggregation of toxin-antitoxin particles. When the same mixtures were placed in larger capillaries they flocculated in the same time as in test-tubes, but it was not so easy to distinguish the order of flocculation of mixtures in which the antitoxin spacing was 10 per cent. In the second place it was not possible to distinguish with certainty between flocculated and non-flocculated mixtures that had been placed in fine capillaries. This was because the fine striations in these tubes, especially when they were slightly out of focus, gave rise to a grey appearance and led to false conclusions as to flocculation.

SUMMARY.

A semi-micro flocculation method is described by which the Lf content of 0·2 ml. of toxin solution can be determined.

We are grateful to Miss Winifred Crawford for competent technical assistance.

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